

THE EXPRESSION OF MAMMALIAN
DRUG METABOLISING ENZYMES
IN S. cerevisiae

STEPHEN M. BLACK

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UNIVERSITY OF EDINBURGH
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DECLARATION

I declare

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author involved is clearly stated.

Adaption to a chemically challenging environment is a critical aspect of the evolutionary process. As a result a wide variety of systems involved in xenobiotic metabolism have evolved. Two such systems are the phase I (drug toxification) Cytochrome P-450 monooxygenases and the phase II (drug detoxification) glutathione S-transferases. In order to evaluate the role of specific gene products in chemical toxicity and mutagenicity members of each of these groups have been expressed in the lower eukaryote S.cerevisiae and the mutation frequency and sensitivity to a wide variety of cytotoxic agents examined.

A cDNA encoding the rat P-450IIB1 protein has been expressed to a level of between 0.1-0.2% of total yeast protein. The protein was localised to the microsomal fraction and found to be functional as determined by activity towards the model substrate, benzyloxyresorufin. An attempt was made to develop this P450IIB1 expressing strain as a viable short term test (STT) for the screening of potential mutagens. To this end the strain was exposed to a series of known mutagens all requiring metabolic activation by cytochrome P-450 to exert their effects. An increase in mutation frequency, as determined by resistance to L-canavanine, was obtained when exposed to the anticancer drug, cyclophosphamide and the mycotoxin, sterigmatocystin. The PB-inducible cytochrome P-450's have been implicated in the metabolism of these chemicals. The specificity and potential of this P450IIB1 expressing strain and the possibilities for the expression of cytochrome P-450's in S. cerevisiae as an STT is discussed.

The overexpression of the glutathione S-transferases (GST) has been associated with drug resistance. In order to directly evaluate this possibility the human alpha (B₁B₁) and pi class GST have been expressed in S. cerevisiae either singly or in tandem. These strains were then exposed to a variety of alkylating agents, anticancer drugs and organic hydroperoxides to determine if GST overexpression modulates sensitivity to cytotoxic insult. In all cases GST expression resulted in a significant reduction in the cytotoxic effects of these agents. These data provide evidence that the overexpression of GST observed in cells resistant to anticancer drugs is directly involved in the resistance mechanism.

ABBREVIATIONS

A	Adenine
2-AAF	2-acetylaminofluorene
Adr	Adriamycin
Ahh	Aryl hydrocarbon hydroxylase
2-AN	2-anthramine
ara-C	1- β -arabinofuranosylcytosine
ATP	Adenosine triphosphate
B(a)P	Benzo(a)pyrene
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
bp	Base pairs
BSO	Buthionine-S,R-sulphoximine
C	Cytosine
Can	L-canavanine
CDNB	1-chloro-2,4-dinitrobenzene
CHO	Chinese hamster ovary cells
Chl	Chlorambucil
CHP	Cumene hydroperoxide
CPA	Cyclophosphamide
CsCl	Caesium chloride
DBE	1,2-dibromoethane
DEM	Diethylmaleate
DEPC	Diethylpyrocarbonate
DHFR	Dihydrofolate reductase
diW	Double distilled water
DNA	Deoxyribonucleic acid

ds	Double stranded
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
FDNB	Fluorodinitrobenzene
G	Guanine
GH	Growth hormone
GHCl	Guanidinium hydrochloride
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S-transferase
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
i.p.	intraperitoneally
IPTG	Isopropyl-1-thio-B-D-galactoside
kb	kilobase pairs
kD	Kilodaltons
MC	3-methylcholanthrene
mdr	multiple drug resistance
min	minutes
MOPS	3-(N-morpholino)propane sulphonic acid
mRNA	Messenger ribonucleic acid
MTX	Methotrexate
B-NA	B-naphthylamine
OD	Optical density
OPT	Ortho-phthaldehyde
P-450	Cytochrome P-450
P-450 reductase	NADPH-dependant cytochrome P-450 reductase

PB	Phenobarbital
PCN	Pregnenolone-16 α -carbonitrile
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
ss	Single stranded
STC	Sterigmatocystin
T	Thymidine
TCA	Trichloro acetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TE	10mM Tris-HCl, pH7.5, 1mM EDTA
TEMED	N,N,N ¹ ,N ¹ - tetramethylene diamine
Top II	DNA topoisomerase II
Tris	Tris (hydroxymethyl) amino ethane
UV	Ultraviolet
Vol	volume
X-gal	5-bromo-4-chloro-3-indocyl-B-D-galactoside

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CHAPTER 1

INTRODUCTION

The following sections are meant to represent a broad overview of the cytochrome P-450 and glutathione S-transferase enzyme systems. A more comprehensive look at the aspects of these enzyme systems of direct relevance to this thesis are to be found at the beginning of Chapters 3 and 4.

1.1 Discovery of Cytochrome P-450

Living organisms are constantly exposed to chemical substances, some necessary for life, some with medicinal value and others of no functional value. The latter two groups are referred to as xenobiotics (Astrom & De Pierre, 1986). Xenobiotics have always been present in the environment, either being formed by natural processes or, more frequently in an industrial society, as by-products of modern industrial concerns. The toxic effects of xenobiotic exposure can be combatted in two ways, either the compound is prevented from entering the body (or cell), and/or is excreted rapidly (Astrom & De Pierre, 1986). Problems arise when the xenobiotic is lipophilic. In these cases the lipid based cell membrane provides no permeability barrier towards xenobiotics and also excretion cannot be carried out via the aqueous solutions of bile or urine.

Thus in the absence of systems to render lipophilic xenobiotics more water-soluble cellular accumulation

would occur until toxic concentrations are reached. Fortunately, systems designed to facilitate the excretion of hydrophobic compounds have evolved. These systems involve oxidation reactions that are predominantly catalysed by the cytochrome P-450 dependent monooxygenases (P-450) (Wolf, 1986).

P-450 was first identified as a reduced carbon monoxide complex with an absorption band at 450nm (Garfinkel, 1958; Klingenberg, 1958). This pigment was further characterised and named cytochrome P-450 (Omura & Sato, 1964). The original function of P-450 remains obscure, however the ancestral protein evolved at a very early time and P-450 can be detected in virtually all organisms including bacteria, yeast, plants, insects and vertebrates. P-450's have probably existed for more than 1.5 billion years (Nebert & Gonzalez, 1987). Various theories have been proposed for the function of the earliest P-450 suggesting that it may have provided a way to utilise otherwise inert molecules in the environment by the incorporation of molecular oxygen (Nebert & Gonzalez, 1985). The oxidation of straight chain alkanes produces alcohols which may have represented an initial step in the formation of fatty acids. These could then have been used either as an energy source or as a component of biological membranes (Nebert & Gonzalez, 1985). P-450 may also have been involved in the

deactivation of reactive oxygen species due to its innate peroxidase activity (Nebert & Gonzalez, 1985).

Alternatively it has been proposed that P-450 may have initially evolved as an oxygen 'sensor' or as an enzyme involved in the synthesis of steroids (Nebert & Gonzalez, 1985).

The P-450 monooxygenase system is located in the endoplasmic reticulum (ER). This system comprises two proteins; the flavoprotein NADPH-dependent cytochrome P-450 reductase (P-450 reductase) which reduces P-450 by a two electron transfer mechanism (White & Coon, 1980). The second electron may also be transferred from cytochrome b_5 (Guengerich, 1979). More than 75% of the P-450 reductase is found to be located free of the lipid bilayer of the ER whereas the P-450 protein itself is found to be embedded in the membrane by the insertion-halt-transfer sequence located at the amino terminal of the protein. The stoichiometry of P-450 reductase: P-450 protein in mammalian liver ranges between 1:10 and 1:100 (Nebert & Gonzalez, 1987).

In general P-450 mediated metabolism results from the insertion of one atom derived from molecular oxygen into C-H bonds. However, dependent on the substrate and the nature of the intermediates, many different reactions are possible as shown in Figure 1.1. Thus various reactions catalysed by P-450 include aliphatic and

aromatic oxidation, N-,O- and S- dealkylations, oxidative deaminations, sulphoxide formation, N-oxidation, N-hydroxylation and oxidative and reductive dehalogenation. Dependent on the rate and stability of reactive intermediates formed by P-450 mediated reactions as well as the presence of Phase II enzymes (epoxide hydrolases, glutathione S-transferases, etc) two pathways exist. There can be further metabolism (e.g. conjugation to glutathione) and the excretion of highly polar species in the bile or urine. Alternatively the reactive intermediates can covalently bind to proteins and nucleic acids within the cell. The second pathway has been shown to be associated with chemical carcinogenesis , mutagenesis , drug toxicity and teratogenesis (Wolf, 1986; Nebert & Gonzalez, 1987; Gonzalez, 1988). It should, however, be noted that not all P-450's are involved in catabolic reactions. P-450's located in the adrenal gland are involved in the formation of steroids. These include two microsomal (steroid 17 α -hydroxylase and steroid 21-hydroxylase) and two mitochondrial (cholesterol side chain cleavage and steroid 11 β -hydroxylase) enzymes (Nebert, 1988).

1.2 Cytochrome P-450 Evolution

The P-450 system has diverged and duplicated extensively from a common ancestral gene. It can now be

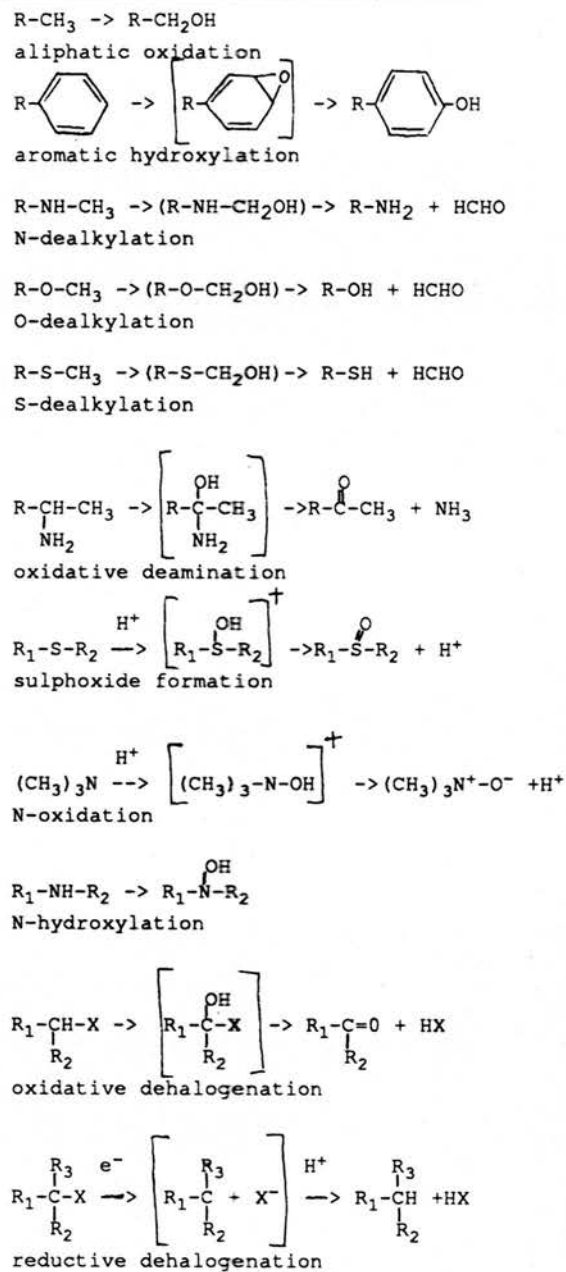


Fig.1.1 Cytochrome P-450 Catalysed Reactions.

Shown are the varied types of reactions catalysed by the cytochrome P-450 monooxygenase enzymes. This figure is reproduced from Nebert & Gonzalez (1987).

regarded as a gene superfamily probably comprising at least 50 genes in mammals (Wolf, 1986). Evolutionary relationships between P-450's can be estimated by comparing amino acid sequences (67 at the end of January, 1987) (Nebert & Gonzalez, 1987). These comparisons can be used to construct phylogenetic trees as shown in Figure 1.2. At present the P-450 supergene family consists of 13 families including two bacterial, two yeast, one insect and eight vertebrate families (Nebert & Gonzalez, 1987). Families I to IV code for microsomal enzymes involved in the formation of complex metabolites while families XVII, XIX, XXI and XXII encode P-450's involved in steroid biogenesis. The mitochondrial P-450's are found in family XI. The P-450 identified in the house-fly is in family VI. In micro-organisms the two yeast P-450's are found in families LI and LII while the bacterial P-450 of Pseudomonas is found in family P450CI and that from Bacillus megaterium represents the CII gene family (Gonzalez, 1988).

A number of conclusions can be drawn from the phylogenetic tree in Figure 1.2. The unit evolutionary period (UEP) can be calculated, i.e. the time in millions of years for a 1% change in amino acid sequence. For P-450's this is found to vary between two and four. Since the UEP is thought to be predictive of the structural constraints within a protein and the need for genetic

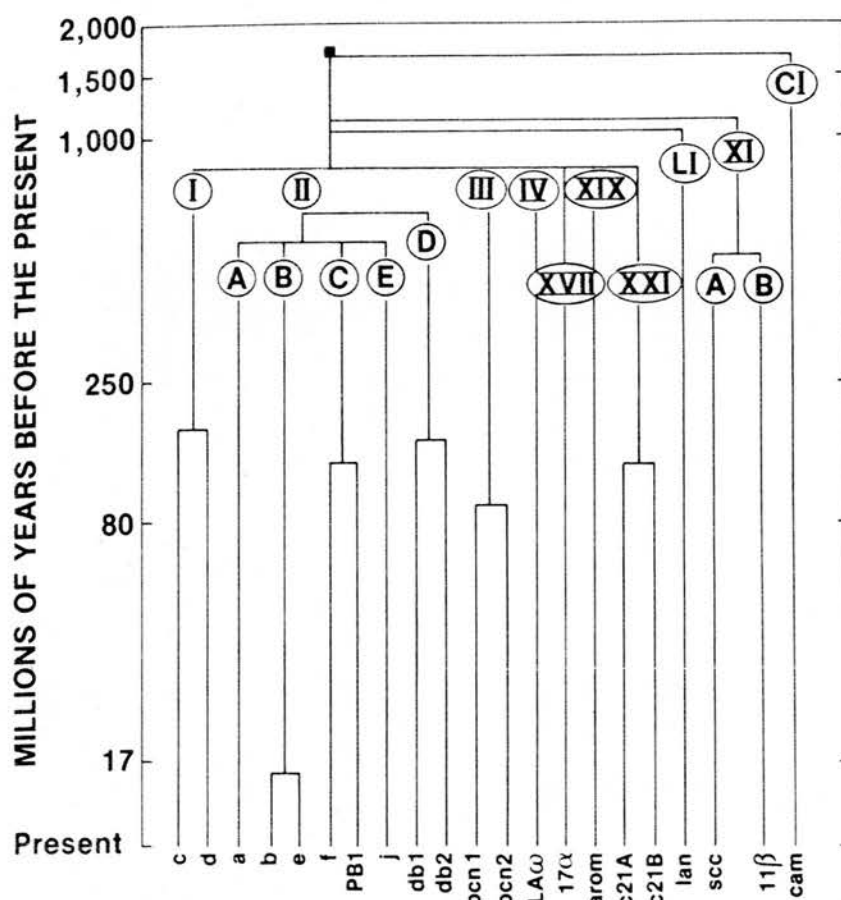


Fig.1.2 Cytochrome P-450 Phylogenetic Tree.

The divergence times given in this phylogenetic tree are calculated according to Nelson & Strobel (1987) using 69 P-450 sequences. A description of the reactions catalysed by the different P-450 families shown are described in the text. This figure is reproduced from Gonzalez (1988).

diversity UEP's can be particularly informative. For example, histones, which are amongst the most stable of all proteins have a UEP of 400 since they require a highly conserved tertiary structure in order to function (Gonzalez, 1988). At the other end of the scale are the immunoglobulins which have a UEP of only 0.7 to allow for a diverse antigen binding capacity (Gonzalez, 1988). Thus since P-450 must evolve to metabolise a wide variety of structurally diverse compounds and, therefore, are required to be very adaptable this explains a very high rate of divergence.

The evolution of the various P-450 enzymes is also illustrated in Figure 1.2. Phylogenetic trees suggest that the early P-450's may have evolved to metabolise cholesterol and its derivatives. Amongst the oldest P-450's are the mitochondrial enzymes in the P450XI family involved in cholesterol metabolism (Nelson & Strobel, 1987). P-450 enzymes involved in cholesterol biosynthesis evolved before the divergence of prokaryotes from eukaryotes since three types of bacteria are known to contain - and presumably synthesise - cholesterol or partially demethylated lanosterol derivatives (Bloch, 1983). Related to these P-450's are those involved in fatty acid metabolism which along with the cholesterol metabolising enzymes may have been involved in the maintenance of membrane integrity in the early eukaryotes

(Nebert & Gonzalez, 1985). A later evolutionary event was probably the endogenous steroid synthesising enzymes and the catabolic enzymes, around 900 million years ago. The latter group have diverged rapidly over the last 400 million years into the major drug and carcinogen metabolising enzymes of the P450I and P450II gene families (Nelson & Strobel, 1987). It has been suggested that the rapid increase in the number of P-450's within certain gene families in the last 400 million years may have coincided with the emergence of aquatic vertebrates on to land and the resultant dietary changes associated with consumption of land vegetation (Nelson & Strobel, 1987).

1.3 Regulation of Cytochrome P-450

It is now becoming apparent that P-450 genes are under complex control, during development, in response to endogenous signals, and following the exposure of the organism to xenobiotics. That P-450 regulation can involve age, sex, diet and endogenous compounds was initially shown by Conney (1967). This is now known to involve sex specific expression, and developmental, transcriptional as well as post-transcriptional regulation. The following paragraphs give examples of the factors known to be involved in the control of P-450 expression during an organisms life-cycle.

1.3.1 Sex Specific P-450 Gene Expression

A variety of mechanisms involving both induction and suppression are involved in the sexual differentiation of P-450's. It has been suggested that the control of P-450 between the sexes is via suppression rather than induction (Gonzalez, 1988). An example of this is seen in P450IIIA mRNA expression where formation is suppressed in female rats at the onset of puberty (Gonzalez, 1988). This decrease in P450IIIA mRNA may be controlled via a corresponding decrease in circulating corticosteroid levels (Waxman et al, 1988). As well as steroid hormone levels growth hormone is known to play a role in sex-specific repression (Gonzalez, 1988). Hypophysectomy reduces circulating growth hormone in male rats which leads to an increase in testosterone 6 β -hydroxylase activity.

However, the pulsatile infusion of growth hormone decreases the level of this enzyme (Yamazo et al, 1986). How steroid and pituitary hormones control the suppression of certain P-450's in a sex-specific manner is as yet unknown.

1.3.2 Developmental Regulation of P-450 Expression

In general most P-450's are not expressed or inducible prior to birth. However, exceptions are the P450IIB and P450IIEI genes of the rat and the P450IA1

gene of the mouse. Rat P450IA1 was found not to be inducible by 3-methylcholanthrene (MC) (Giachelli et al, 1987). Why there should be species differences in developmental regulation as well as the factors controlling prenatally inducible P450's remain as yet undiscovered.

1.3.3 Transcriptional regulation of Cytochrome P-450 Expression

The most studied P-450 with respect to transcriptional regulation is the MC/TCDD induction response. Exposure to MC causes an increase in aryl hydrocarbon hydroxylase activity in rats. The use of metabolic inhibitors has shown that this response was due to transcriptional activation (Gelboin & Blackburn, 1963). The analysis of this system has delineated the types of transcriptional regulation occurring in P-450 expression. Mostly this work has been carried out using P450IA1 promoter - CAT constructs. Among the elements found were an inducible enhancer, found in multiple copies and named the xenobiotic regulatory element (XRE) (Fujisawa-Sehara et al, 1987). This has been shown to be 15 nucleotides in length and to bind nuclear proteins (Fujisawa-Sehara et al, 1987). Within the same region of the P450IA1 promoter there is also a drug-regulatory element (DRE) separate from the XRE (Gonzalez, 1988).

Closer to the transcriptional start site, an element that was found to suppress constitutive expression from the P450IA1 promoter has been discovered (Hines et al, 1988). Thus P-450 regulation at the level of transcription can involve both induction and suppression.

1.3.4 Post-Transcriptional Regulation of Cytochrome P-450 Expression

Post-transcriptional regulation can occur at the level of mRNA or protein and both are used to control P-450 expression. For example, P450IA2 mRNA is stabilised in the liver and extrahepatic tissues by exposure to TCDD (Gonzalez, 1988). P450p mRNA is also stabilised by the antibiotic TAO (Watkins et al, 1986). TAO has also been shown to decrease P450p protein degradation (Watkins et al, 1986). Perhaps the best example of post-transcriptional regulation is seen with the P450IE1 enzyme. The administration of small organic compounds such as acetone, pyrazole and ethanol cause a rapid induction in P450IE1 protein while leaving mRNA levels unaffected. Studies using in vivo pulse labelling with amino acids ruled out the possibility of an increase in translation but suggested that the protein itself was being stabilised (Gonzalez, 1988).

1.4 Cytochrome P-450 Expression and Carcinogenesis

The response of an organism to a drug is dependent on its rate of metabolism. This metabolism can lead to drug deactivation, or in some cases lead to activation, cyclophosphamide for example. Thus the polymorphic expression of P-450 in humans leads to altered drug metabolism which is of considerable pharmacological and toxicological relevance (Wolf, 1986). The polymorphic expression of P-450 has been shown to alter susceptibility to environmental toxins and carcinogens.

For example, the difference in P450IA1 levels in mice is associated with an increase in carcinogen-induced sarcomas, skin and lung carcinomas, lymphomas and leukaemias, such that a higher expression of P450IA1 protein increases susceptibility (Nebert & Jensen, 1979). It has been postulated that the expression of P450IA1 may be important in a variety of human cancers, such as those of the lung, larynx, kidney and cancers of the urinary tract (Nebert & Jensen, 1979). However, studies to date have been contradictory and the role of P450IA1 expression is still unclear (Wolf, 1986).

In man there also appears to be polymorphisms associated with the P450IIC and P450IID gene families. There have been suggestions that the level of steroid hormone catabolism within an organism can be related to cancer susceptibility. These reactions are of importance

where circulating hormone levels have been shown to play a role in tumour development, for example endometrial and breast cancers (Wolf, 1986). In the human population the 16 α - hydroxylation of estradiol (an activity associated with the P450IIC family) has been linked with the incidence of mammary tumours (Bradlow et al, 1985). It should, however, be noted that P-450's other than the P450IIC protein can catalyse the 16 α -hydroxylation reaction and thus may also be implicated in the susceptibility of the organism to cancers of this type. The polymorphism associated with P450IID1 expression relating to debrisoquine metabolism has been extensively studied (Gonzalez et al, 1988). The homozygous poor metabolizers, as well as the heterozygotes appear to have a considerably reduced risk of lung and liver cancer (Ayesh et al, 1984). It should be noted, however, that the studies which suggest that polymorphic P-450 expression alters cancer susceptibility are few and produce questionable data. For example, the P450IID1 locus was linked to drug-induced Parkinsons disease (Borbeau et al, 1984). However little emphasis is now placed on this study due to the lack of proper controls. Xenobiotic exposure without doubt plays an important role in cancer development. Thus the polymorphic expression of P-450 may play an important role in those cancers in which xenobiotic exposure plays a part. However, the studies to date are far from conclusive.

1.5 Cytochrome P-450 Expression in S.cerevisiae

Cytochrome P-450 was initially detected in mammalian liver microsomes. Its presence in other organelles, tissues and species was unclear. However, it was established that P-450 were expressed in many tissues and also in various vertebrates, invertebrates, plants and also micro-organisms. This included the discovery of the P-450 system in S.cerevisiae and in 40 other microbial systems (Kapelli, 1986). Lindenmayer & Smith (1964) initially demonstrated that cellular extracts of S.cerevisiae contained P-450. The formation of P-450 in the yeast S.cerevisiae has since been characterised as a protein associated with growth in glucose (Wiseman et al, 1978; Blatiak et al, 1985). S.cerevisiae P-450 levels are, therefore, limited by the physiological state of fermentation (Kapelli, 1986). Wiseman & King (1982) have suggested that any one of four factors is required for optimal P-450 synthesis in S.cerevisiae:-

- 1) A concentration of glucose between 4 and 20%.
- 2) Anaerobic growth conditions (decreases mitochondrial cytochrome synthesis).
- 3) Inhibition of mitochondrial protein synthesis (which may increase the heme pool).
- 4) Use of respiratory deficient mutants.

These factors suggest an inverse correlation between mitochondrial cytochrome and cytochrome P-450 content. This has been shown to be the case (Kapelli, 1986).

1.5.1 Physiological Role of Cytochrome P-450 in S.cerevisiae

It has been shown that P-450 in S.cerevisiae is involved in the late stages of ergosterol biosynthesis (the major yeast membrane sterol). Ohan et al (1978) have shown an oxygen and NADPH-dependent conversion of lanosterol to 4,4 dimethyl zymosterol in S.cerevisiae. This can be inhibited by antibodies raised against P-450. Aoyama and Yoshida (1978) then demonstrated that, in a reconstituted system, yeast P-450 could oxidatively remove the 14 α -methyl group of lanosterol.

A second P-450 in S.cerevisiae has been demonstrated by Hata and co-workers (1983) who have shown the presence of a Δ^{22} - desaturation reaction of ergosta-5,7-dien-3 β -ol. This reaction is still observed in the 14 α - demethylase deficient strain, SG1. The desaturation reaction had reduced activity in the mutant strain N22. However, the 14 α -demethylation activity was normal (Hata et al, 1983).

Aoyama et al (1978) have also purified the NADPH dependent cytochrome P-450 reductase (yeast reductase) from yeast microsomes. This protein was shown to give a

functional monooxygenase system in a reconstituted assay. However, more importantly, the purified yeast reductase was shown to couple with rabbit liver P-450 to catalyse benzphetamine N-demethylation. This provided direct evidence for the coupling of a mammalian P-450 to yeast reductase to produce a functional monooxygenase system. An essential requirement if a mammalian P-450 expressed in yeast is to be active.

1.5.2 S.cerevisiae in Mutagenicity Testing

Many studies have shown that mutation assays using yeasts can be used to predict carcinogenic potential and that these assays compare favourably with other eukaryotic test systems in identifying chemical mutagens (Moustacchi et al, 1986). Yeast can also detect a number of mutagens that are negative in prokaryotic mutation tests, for example paraffins, olefins and actinomycin D. They are also inexpensive to perform (Moustacchi et al, 1986). To date, 492 chemicals have been evaluated in S.cerevisiae (Zimmerman et al, 1984) together with 41 other industrial compounds (Dean et al, 1985) and 20 compounds identified in pulp mill effluents (Nestmann & Lee, 1985).

The bioactivation of promutagens has been shown to occur in S.cerevisiae (Callen & Philpot, 1977; Callen et al, 1978; Callen et al, 1980; Kelly & Parry, 1983; Kelly et al, 1985) with a correlation between high cellular P-

450 levels and activation. The main question in the use of S.cerevisiae is whether the P-450 system produces the same metabolites as a hepatic S9 fraction. It is also possible that the activation observed in yeast does not only occur via a P-450 mediated reaction (Kapelli, 1986). Other problems are that yeast are often less sensitive than bacteria in terms of the minimal chemical concentrations required to elicit a response. This is probably due to very powerful cellular detoxification processes as well as the tough outer cell wall (Moustacchi et al, 1986). However the cloning and expression of different mammalian P-450 cDNAs offers the possibility of constructed yeast strains with a specific and well characterised activation system. Were this to occur it would make S.cerevisiae a very useful organism for short-term mutagenicity tests.

1.6 Glutathione S-transferases

As mentioned previously (Section 1.1) certain compounds present in the environment play a central role in the development of cancer. These include drugs, food additives, industrial effluents, pesticides and cigarette smoke (Ames, 1979; Chasseaud, 1979). When humans are exposed to xenobiotics they can be converted by a variety of cellular reactions into products that are more, less or equally toxic as the parent compound. For example, the

oxidation reactions carried out by P-450 can produce carcinogenic species from chemicals that are otherwise chemically inert (Nebert & Gonzalez, 1985; Wolf, 1986; Gonzalez, 1988). P-450 is an example of a phase I (toxification) enzyme but present within the cell are a variety of phase II (detoxification) enzymes which include the glutathione S-transferases (GST). The action of GST in a conjugation reaction with the thiol compound glutathione (GSH) generally produces products that are less toxic to the cell than the parent compound.

GST were first identified by Booth, Boyland & Sims (1961) where a partially purified liver enzyme was shown to catalyse the formation of GSH derivatives from various compounds. It was later suggested that the role of GST and GSH was in the protection of cellular constituents from the damage caused by electrophilic agents (Boyland & Chasseaud, 1969). In the reactions catalysed by GST, the sulphur atom of GSH donates electrons for the nucleophilic attack on, or reduction of, an electrophilic substrate. The GSH conjugate can either be excreted directly or further metabolised (Mannervik, 1985). Further metabolism, via hydrolysis, results in an S-(chemical)-cysteine derivative which is subsequently N-acetylated to yield a mercapturic acid, the normal excretion product of GSH conjugates in the urine (Mannervik, 1985).

There are multiple forms of GST which have been shown to be present in all organisms so far examined. Chromatographic and electrophoretic measurements along with activity measurements using the 'general' substrate 1-chloro-2,4-dinitrobenzene (CDNB) has led to the discovery of GST in man, rat, mouse, hamster, guinea pig, chicken, cow, monkey, sheep, trout, shark, little skate, grass grub, house-fly, american cockroach, six species of earthworm, pea seedlings and corn (Mannervik, 1985). However, it has been reported that GST is not present in yeast (Jakoby, 1978). The existence of multiple GST isoenzymes within an organism and its conservation through evolution suggests an important biological function (Mannervik, 1985). If, as suggested, detoxification is the major role of GST then the presence of several forms may allow for a broader range of substrate specificity than would otherwise be possible, and therefore, GST can be found as both cytosolic and microsomal forms. The cytosolic forms will be dealt with in detail here. The cytosolic and microsomal forms of GST act either as dimeric or trimeric proteins respectively with each subunit possessing its own distinct active site. The ability of the cytosolic enzymes to form dimers does not appear to influence catalytic activity (Mannervik, 1988). There is little information on the active site structure within the GST

enzyme. However, each site has a binding site for GSH and an adjacent, partly hydrophobic, binding site for the electrophilic substrate. These subsites within the active site cavity have been referred to as the G- and H-sites respectively (Mannervik et al, 1978). It has been demonstrated that GST have high specificity for GSH as the active thiol suggesting very strict steric requirements for binding to the G-site (Habig et al, 1974). It is likely that the three charged groups of the GSH molecule are bound to amino acid side chains of opposite charge in the GST protein (Mannervik, 1985). The H-subsite seems to display a great deal of flexibility to accommodate a wide variety of chemical groups as the electrophilic substrate. For example, rat GST 3-3 (μ class enzyme) acts on both 1,2-dichloro-4-nitrobenzene and bromosulfophthalein, the first is uncharged while the second is both negatively charged and a much larger molecule (Habig et al, 1974).

1.7 GST Nomenclature

Since their initial discovery (Booth et al, 1961) a variety of nomenclatures have been used: glutathione S-alkyltransferase, glutathione S-aryltransferase, glutathione S-aralkyltransferase, glutathione S-alkenyltransferase, glutathione S-epoxidetransferase, nitrate ester reductase, steroid

isomerase, Δ^5 -3-ketosteroid isomerase and ligandin (Jakoby, 1978). These titles were based on the erroneous assumption that unique enzymes are responsible for each activity (Mannervik, 1985).

However, comparison of the specific activities of purified GST with a variety of substrates demonstrated that all GST isoenzymes have broad and overlapping substrate specificities. This nomenclature was thus inadequate to designate GST isoenzymes.

A new nomenclature has been proposed using numbers to indicate subunit composition (Jakoby et al, 1984). Thus each GST dimer is identified on the basis of the numbers given to its two constituents (Jakoby et al, 1984). Subsequently each of the proteins have been classified, on the basis of sequence homology, into three gene families termed alpha, mu and pi (Mannervik & Danielson, 1988). Most work has been carried out in the rat with the nomenclature shown in Table 1.1.

At present human GST have been denoted by Greek letters. At present two enzymes with basic isoelectric points have been referred to as GST B₁ and B₂ (Stockman et al, 1987). An enzyme with near neutral isoelectric point was named GST μ and acidic proteins from erythrocytes or placenta GST λ or pi respectively (although these may in fact be the same protein) (Mannervik, 1985). Thus in humans at least three

NOMENCLATURE FOR RAT CYTOSOLIC GLUTATHIONE S-TRANSFERASES

<u>New nomenclature</u>		<u>Previous nomenclature</u>	
Glutathione transferase	1-1	B (ligandin)	Y_a
Glutathione transferase	1-2		Y_a, Y_c
Glutathione transferase	2-2	AA	Y_c
Glutathione transferase	3-3	A	Y_b^1
Glutathione transferase	3-4	C	Y_b^1, Y_b^2
Glutathione transferase	3-6	P	
Glutathione transferase	4-4	D	Y_b^2
Glutathione transferase	4-6	S	
Glutathione transferase	5-5	E	
Glutathione transferase	6-6	M_T	Y_n
Glutathione transferase	7-7	P	Y_p
Glutathione transferase	8-8	K	Y_k or Y_f

Table 1.1 Rat Glutathione S-transferase Nomenclature.

The new nomenclature proposed for the rat cytosolic glutathione S-transferases is shown.

Also included are the nomenclatures previously used for these enzymes. This table is reproduced from Mannervik & Danielson (1988).

distinct groups exist termed: alpha (B_1B_1 , B_1B_2 , B_2B_2) mu (μ) and pi (λ/π). This nomenclature is shown in Table 1.2.

1.8 Characterisation of Human GST

Since the studies presented in chapters 4 and 5 involve human alpha and pi class GST, the following gives a brief outline of the diagnostic substrates for each of the three classes (alpha, mu and pi). All classes also exhibit high activity towards the 'general' substrate CDNB.

1.8.1 Human Alpha Class GST

This class of GST isoenzyme has characteristically high selenium-independent glutathione peroxidase activity with organic hydroperoxides, such as cumene hydroperoxide. Also this class of human enzyme has high isomerase activity with Δ^5 -androstene-3,17-dione as substrate (Mannervik, 1985). The dimeric protein has an Mr of approximately 51,000. Alpha class GST appear to be abundant in all human livers.

1.8.2 Human Mu Class GST

A diagnostic substrate for this class of GST is trans-4-phenyl-3-butene-2-one while other high activity substrates are trans stilbene oxide, styrene-7,8-oxide

CHARACTERISTICS OF THE HUMAN CYTOSOLIC GLUTATHIONE S-TRANSFERASES

<u>Isoenzyme</u>	<u>Class</u>	<u>Subunit Mr(KD)</u>	<u>Isoelectric point</u>
B ₁ B ₁	Alpha	25	8.9
B ₁ B ₂	Alpha	25	8.75
B ₂ B ₂	Alpha	25	8.4
μ	Mu	26.5	6.6
ψ	Mu	26.5	5.5
π	Pi	23	4.8
Skin"9-9"	Alpha	27.5	9.9

Table 1.2 Human Glutathione S-transferase Nomenclature.

The class and subunit composition of the human cytosolic enzymes is given. Also included are the molecular weights (kD) of each subunit and their isoelectric points. This table is reproduced from Mannervik & Danielson (1988).

and benzo(a)pyrene-4,5-oxide (Mannervik, 1985). The dimeric protein has an Mr of approximately 53,000. Genetic studies using liver samples from Chinese, Indian and Caucasian subjects has demonstrated that a gene within the mu locus in humans is polymorphic and the protein is expressed in only 60% of subjects (Ketterer, 1988).

1.8.3 Human Pi Class GST

GST pi can be characteristically diagnosed using the substrate ethacrynic acid (Mannervik, 1985). The Mr of the dimeric GST is approximately 47,000. Although possessing no selenium independent glutathione peroxidase activity towards cumene hydroperoxide GST pi does have considerable such activity with linoleate hydroperoxide as substrate (Ketterer, 1988). Linoleate hydroperoxide is thought to be a breakdown product formed by damage to biological membranes.

1.9 Glutathione (GSH)

The tripeptide γ -glutamyl cysteinyl glycine, glutathione is one of the most abundant small organic molecules in nature (Meister & Anderson, 1983). GSH is synthesised intracellularly by the consecutive actions of γ -glutamyl cysteine synthetase (reaction 1) and GSH synthetase (reaction 2). Reaction 1 is negatively

feedback regulated by GSH itself. GSH can also be degraded (reaction 3) by γ -glutamyl transpeptidase. The turnover of GSH is controlled by the activity of γ -glutamyl transpeptidase the location of which is restricted and specific (Hanigan & Pitot, 1985).

Most tissues contain GSH with intracellular levels being as high as 15mM in the lens and 5-10mM in the liver (Ketterer, 1988). It should be noted that GSH need not necessarily be found in every cell in a tissue in which it is present. For example, although the overall concentration of GSH in the brain is 1.5 - 2 mM histochemical studies show no GSH present in the neuronal stroma (Ketterer, 1988).

1.10 Influence of GSH on Antineoplastic Therapy

GSH metabolism can exert an influence on antineoplastic therapy in four ways as detailed below:-

1.10.1 GSH and Detoxification of Anticancer Drugs

GSH is involved in the detoxification of two classes of anticancer drug, electrophilic agents and those drugs which generate reactive oxygen species (Wolf et al, 1987b). The first type represent the alkylating type of anticancer agent including cyclophosphamide, chlorambucil and melphalan. The second are those which yield hydrogen peroxide and free radicals such as the

superoxide anion (Hayes & Wolf, 1988). Many of the latter group are the quinone-containing anticancer drugs such as adriamycin, daunorubicin and mitomycin C. Thus the intracellular concentration of GSH within normal and tumour cells will be an important factor in determining therapeutic efficacy (Wolf et al, 1987b).

1.10.2 GSH and Toxification of Anticancer Drugs

GSH is known to cause toxification of anticancer drugs in a limited number of cases, these being azathiopurine and bleomycin. The formation of the GSH-imidazole adduct is believed to be the major route of azathiopurine toxification (Arrick & Nathan, 1984). Bleomycin forms a complex with ferrous iron that can react with molecular oxygen in such a way that an oxidised ferric iron-bleomycin complex is formed which also results in the formation of superoxide anion (Caspary et al, 1981). It has been proposed (Caspary et al, 1981) that the binding of GSH to the ferric iron-bleomycin complex results in the reduction back to the active ferrous iron state. Thus a catalytic oxidation-reduction cycle is produced in which reducing equivalents from GSH are ultimately transferred to molecular oxygen to form superoxide anion (Caspary et al, 1981).

1.10.3 Role of GSH in Anticancer Drug Delivery

The sole example of GSH modifying the delivery of a drug is with methotrexate. The uptake of methotrexate by cultured rat hepatocytes is accelerated by the addition of GSH to the medium (Leszczynska & Pfaff, 1982). Obviously GSH dependent metabolism and excretion of anticancer drugs ultimately affects delivery to the target cell but the influence of GSH on drug uptake, as suggested by methotrexate, may represent another fundamental role for GSH in antineoplastic therapy.

1.10.4 Alteration of GSH Levels by Anticancer Drug Treatment

It is known that the exposure of an animal to a low dose of a cytotoxic compound has the potential to protect against a normally lethal dose of the same compound (Millar et al, 1975). Studies in which cyclophosphamide has been used to 'prime' the bone marrow cells of mice has shown that maximum protection against a lethal dose of cyclophosphamide coincides with peak GSH levels (Adams et al, 1985) such that GSH levels in the primed bone marrow cells were between 1.8- and 3-fold higher than controls. Cancer chemotherapy depends on a narrow therapeutic index thus the over-production of GSH could lead to clinical drug resistance within a tumour. This could lead to a tumour that is refractory to further therapy (Carmichael et al, 1986).

1.11 Physiological Roles of GST

1.11.1 Catalytic Role

On the basis of their catalytic capabilities and high concentration (up to 10% of the cytosolic protein in rat liver) GST appear to be closely involved in the detoxification of ingested compounds.

As previously stated the substrates of GST, both xenobiotics and normal dietary constituents, can be converted to less toxic species when conjugated to GSH. These conjugates whether excreted directly into the bile or, after further metabolism by the kidney, removes potentially harmful compounds from the host. Thus an important question is whether the function of GST is to detoxify the xenobiotics to which the organism is exposed or whether there are also endogenous substrates for GST? The formation both in vivo and in vitro of GSH conjugates of steroids suggests the latter is indeed the case. GSH conjugates of the reactive metabolites of estradiol 17 β and 2-hydroxyestradiol-17 β have been shown to form in vivo (Mannervik, 1985). Some prostaglandins can also serve as substrates for purified GST (Chaudhari et al, 1978). The epoxide derivative of arachidonic acid, leukotriene A₄ methylester, is also conjugated to GSH by purified GST (Mannervik et al, 1984). Leukotrienes constitute the 'slow reacting substance of anaphylaxis' a presumed mediator of allergic and anaphylactic reactions.

Mannervik et al (1984) found that the three human classes of GST differed significantly in their catalytic activity. The pi class GST showed limited ability to convert leukotriene A₄ methylester while the alpha and mu classes catalysed almost complete conversion of the substrate into leukotriene C₄ mono methylester. Thus GST seem to play an important role in the metabolism of steroids and leukotrienes suggestive of a fundamental role in cellular processes.

Cholesterol α -oxide, a product of lipid peroxidation, has also been identified as a substrate for GST (Meyer & Ketterer, 1982). This and the peroxidase activity of GST suggests that GST may play an important role in the protection of biological membranes from lipid peroxidation. This may be of relevance in the protection of cells from the effects of anticancer drugs such as adriamycin whose peroxidative potential is known to damage biological membranes (Tritton & Lee, 1982).

1.11.2 Role as Ligandin

In 1969 Arias and coworkers isolated a protein from rat liver which could bind bilirubin (Levi et al, 1969). They named this the Y protein. Marey & Litwack (1969) independently isolated a protein with an affinity to bind a metabolite of cortisol while Ketterer et al (1967) had previously isolated a protein that could

covalently link the dye butter yellow (4-dimethyl aminobenzene) which he named azo-dye carcinogen binding protein. The use of antibodies against the Y protein established that all three groups had isolated the same protein which was renamed ligandin (Litwack et al, 1971). Ligandin was identified as a 45kD, basic protein composed of two subunits. Habig et al (1974) subsequently showed it to be identical to rat GST B (an alpha class protein). The term 'ligandin' was used to describe its affinity for a wide range of ligands of a hydrophobic character (Jakoby, 1978). These include polycyclic aromatic hydrocarbons, steroids, heme and its derivatives, a variety of drugs including lipophilic antibiotics and thyroid hormones. In fact the number of ligands bound suggests that Ligandin, and to a lesser extent other GST, may serve as an intracellular binding protein on as broad a scale as albumin performs that function extracellularly (Jakoby, 1978).

1.11.3 Covalent Binding

Since many of the substrates for GST are vigorous alkylating agents it is not surprising that some react directly with GST to form stable covalent linkages, for example CDNB. Thus any compound with a strong electrophilic centre will be able to react with the proteins with which it comes into contact (Jakoby, 1978).

If the reagent becomes bound to GST then an interaction with a nucleophilic group on the protein, rather than with DNA, is greatly increased. It should be remembered that ligandin was initially isolated as an azodye carcinogen binding protein capable of forming a covalent linkage to the azodye butter yellow (Ketterer et al, 1967). A large number of procarcinogens are oxidised by cytochrome P-450 to produce highly reactive electrophilic reagents which are potent carcinogens (Wolf, 1986). All are capable of being deactivated by covalent linkage to GST (Jakoby, 1978).

1.12 GST and Drug Resistance

In carcinogenesis, GST are of interest for several reasons. They may confer an advantage of preneoplastic cells over their normal counterparts. They may confer resistance to chemotherapeutic drugs and may also have diagnostic value as tumour markers. The role of GST in drug resistance will be dealt with in detail in Chapter 4, however, all three classes of GST have been implicated in this phenomenon.

GST pi (Cowan et al, 1986; Batist et al, 1986), GST alpha (Robson et al, 1987); Lewis et al, 1988) and GST mu (Smith et al, 1989) have all been shown to be over-expressed in cell lines made resistant to cytotoxic drugs. A microsomal GST has also been implicated in

resistance to nitrogen mustards (Clapper & Tew, 1989). In vivo studies have shown a change in GST and GST related enzymes before and after the onset of clinical drug resistance (Wolf et al, 1987). Also GST have been implicated in rat hepatocarcinogenesis (Farber, 1984a).

1.13 Aims of Thesis

The aim of this thesis was to examine the mutagenic and cytotoxic consequences when mammalian drug metabolising enzymes in the form of cytochrome P-450 or glutathione S-transferases were expressed within the lower eukaryote S.cerevisiae. As an example of the P-450 system a cDNA coding for the rat P450IIB1 (PB_{3a}) protein was expressed. The resultant recombinant strain was then to be evaluated for its potential in short-term mutagenicity testing. Secondly cDNAs coding for members of the human alpha (B₁B₁) or pi class GST were expressed in S.cerevisiae to determine if GST play a role in altering cellular susceptibility to cytotoxic drugs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Microbial Strains

2.1.1 Bacterial Strains

E.coli DH1 (F-recA1, gyrA96, thi-1, hsdR17, supE44)

This strain was used as a recipient for pBR322 based vectors since it is a good host for large scale growth and purification of plasmids (Bolivar et al, 1977).

E.coli K-12 JM101 (thi, supE, (proAB-lac) F'(traD36 proAB lacZ M15I^q))

This strain was used as a host for the pUC based vectors which contain the β -galactosidase gene. In the presence of IPTG, X-gal, and ampicillin a JM101 colony carrying such a vector appears blue. However, the β -galactosidase gene can be disrupted by the insertion of DNA into the multiple cloning sites of these vectors.

The resultant bacterial colonies though still resistant to ampicillin appear white when exposed to IPTG and X-gal.

2.1.2 Yeast Strains

1. S.cerevisiae RH971 (a, leu2, his4, trp1)
2. S.cerevisiae 1.103-5D (α ,leu2- 3/112, ura3-52, trp1, suc5, mal0)
3. S.cerevisiae 1.1-6B (α ,leu2- 3/112, ura3-52, trp1, mal0 suc0)

4. S.cerevisiae ENYWA-1A (α , leu2-3/112; ura3-52,
trp1, his2- Δ 1, SUC3, MAL3, MAL2-8^C)
5. S. cerevisiae YS18 (α , his3-11, his3-15, leu2-3/112,
CanR, ura3-5, trp1)
6. S.cerevisiae UTL7A (α , leu2-3/112, ura3-52, trp1)

The above strains were a gift from Dr A. Hinnen, Ciba-Geigy Biocentre, Basle, Switzerland. They all have trp1 genotype required for the pMA56 expression vector.

7. S.cerevisiae KY118 (α , his3-200, lys2-801^{am}, ade2-101^{OC}, trp1 Δ 1, ura3-52)

This strain was used as a recipient for the expression vectors used in this study. This was principally because the strain was found to have extremely low (almost undetectable) endogenous cytochrome P-450 levels. This strain was a gift from Dr J.D. Beggs, Dept. Molecular Biology, University of Edinburgh.

2.2 Microbial Culture

2.2.1 E.coli Culture Media

L-broth/litre: 10g tryptone (DIFCO), 5g Yeast Extract (DIFCO), 5g Sodium chloride (Sigma).
2g/l glucose (BDH chemicals) was added as a supplement for plasmid growth. (L-Agar contains in addition 15g/l agar (DIFCO). 2xYT/litre 16g tryptone, 10g Yeast extract, 5g Sodium chloride, pH7.0

Terrific Broth/litre: 12g tryptone, 24g Yeast extract, 4.0 ml Glycerol (Sigma), 100 ml 0.17M Potassium dihydrogen orthophosphate; 0.72M di-Potassium hydrogen orthophosphate.

Antibiotics

A stock solution (50mg/ml) of ampicillin (Sigma) was prepared on sterile d1W and stored at -20°C. For cultures containing both pUC and pBR322 plasmids ampicillin was used at a final concentration of 50ug/ml for L-broth and 2xYT and 25ug/ml for Terrific Broth.

2.2.2 Yeast Culture Media

YPDA/litre: 10g Yeast extract (DIFCO), 10g Peptone (DIFCO), 20g Glucose (BDH), 20mg Adenine Sulphate (Sigma) (Adenine sulphate although only required for ade strains was always included). (YPDA agar contains in addition 20g/l agar). Yeast minimal medium/litre 6.7g Yeast Nitrogen base without amino acids (DIFCO), 20g Glucose, 20mg amino acids and vitamins as required to give supplemented synthetic SD medium.

Supplemented synthetic SD medium plus casamino acids/litre 6.7g Yeast nitrogen base without amino acids, 20g glucose, 10g casamino acids, 20mg vitamins as required. Supplemented synthetic SD agar contains in addition 20g/l agar.

2.3 Plasmids

The plasmids used in this study were the general cloning vector pUC-9 and the yeast expression vectors pMA56 and pVT100U (Figure 2.01).

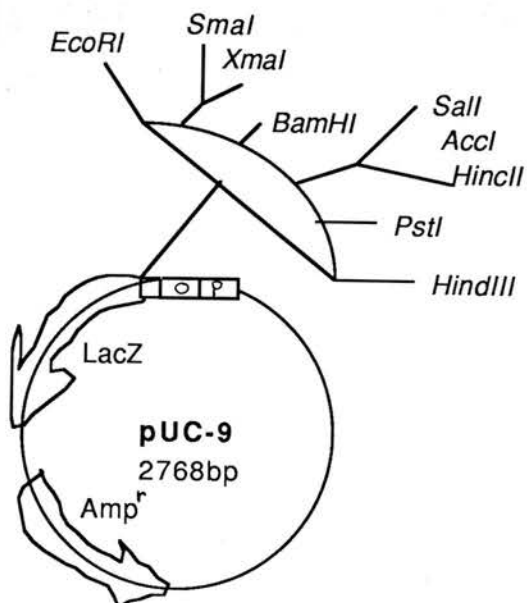
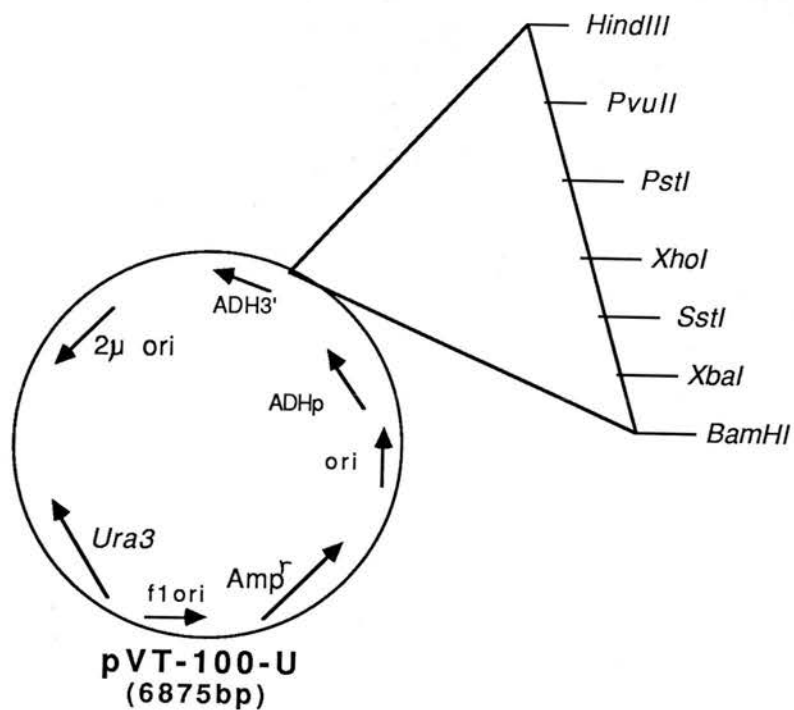
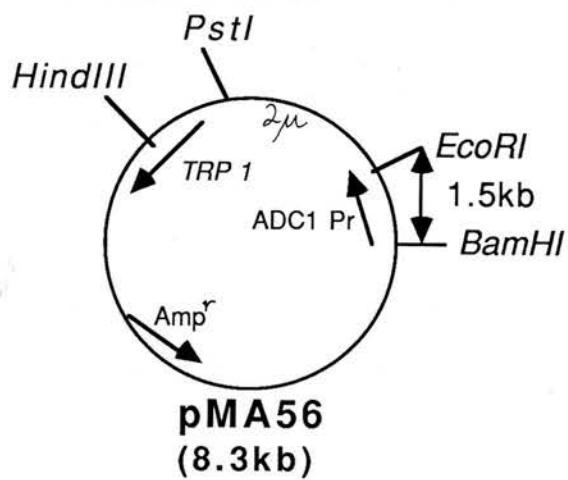
2.4 DNA Analysis

2.4.1 Small Scale Preparation of pUC DNA

The lithium chloride (LiCl) method was used (Willimzig, 1985). L-broth (3ml) or 2xYT broth (3ml) containing 50µg/ml ampicillin was inoculated from a single bacterial colony and grown at 37°C overnight in an orbital shaker (200 rpm). The culture was pelleted sequentially in a 1.5ml eppendorf tube and the supernatant discarded. The pellet was resuspended in 400µl of TELT (50mM Tris-HCl(pH7.5), 62.5mM EDTA, 2.5M LiCl, 0.4% W/V Triton x100). To this was added 40µl of 100mg/ml fresh lysozyme in TELT. The sample was then boiled for 90 sec and cooled for 10 min on ice to precipitate rRNA and protein. The precipitate was pelleted by an 8 min centrifugation in a microfuge and the supernatant removed to a tube containing 0.6 volumes of isopropanol. This was immediately placed at 4°C for 10 min. After a further centrifugation the pellet was washed with 70% ethanol, dried, and resuspended in 40µl of TE (10mM Tris-HCl pH7.5, 1mM EDTA). The plasmid DNA was tested by restriction enzyme analysis and agarose gel electrophoresis.

Fig.2.01 Restriction Maps of the Vectors Used in This Study.

The yeast expression vectors pMA56 and pVT100-U contain the 2 micron origin of replication and the constitutive alcohol dehydrogenase promoter, ADC1. In addition pMA56 contains TRP1 as a selectable marker in S.cerevisiae and pVT100-U URA3. The pUC-9 vector used for the sub-cloning of the rat P450IIB1 cDNA is also shown.



2.4.2 Large Scale Preparation of Plasmid DNA

Single bacterial colonies were picked and transferred to L-Broth (10ml) supplemented with ampicillin (50µg/ml) and the resulting overnight cultures used to inoculate 500ml of Terrific Broth again containing ampicillin (25µg/ml). This culture was incubated at 37°C overnight in an orbital shaker.

Cells were harvested by centrifugation at 5,000 rpm for 10 min and the cell pellets resuspended in 2ml of GTE (50mM glucose, 2mM Tris-HCl pH8.0, 10mM EDTA). To this was added 8ml of a fresh solution of lysozyme (10mg/ml) in GTE and the solution incubated on ice for 30 min. 20ml of fresh alkaline SDS (0.2M NaOH, 1% SDS) was added and after a further 10 min incubation on ice 15 ml of high salt solution (3M potassium acetate, 2M acetic acid) was added. The white precipitate formed was removed after 30 min on ice by centrifugation at 8,000 rpm for 10 min. The supernatant was then filtered through muslin and the DNA precipitated by a 20 min incubation at -20°C after the addition of 2 volumes of 100% ethanol. The precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4°C and resuspended in 6ml of acetate MOPS (0.1M sodium acetate, 0.05M MOPS pH8.0). DNA was again precipitated by the addition of 2 volumes of 100% ethanol followed by a 20 min incubation at -20°C. A centrifugation at 10,000 rpm

for 10 min was used to collect the DNA precipitate. The DNA was resuspended in 10ml of TE and the E.coli genomic DNA and rRNA differentially precipitated by the addition of 1/3 volume 7.5M ammonium acetate and a 20 min incubation on ice. This was then centrifuged at 12,000 rpm for 20 min and the pellet discarded. 2 volumes of 100% ethanol was then added to the supernatant and incubated at -70°C for 60 min. The precipitate (consisting of plasmid DNA, RNA and lipopolysaccharide) was collected by centrifugation and resuspended in 2ml of TE containing 50mM NaCl and 0.1% SDS. RNAase A (DNAase free) (BDH) was added (100µg/ml) and the solution incubated at 37°C for 15 min. The SDS concentration was increased to 1% and proteinase K (BDH) added (100µg/ml) after a one hour incubation at 37°C. The DNA was precipitated by the addition of 1/10 volume 3M sodium acetate (pH4.8) and 2.5 volumes of 100% ethanol (60 min incubation at -70°C). DNA was collected by centrifugation and then resuspended in 3.21ml TE, 3.55g CsCl and 340ul ethidium bromide (10mg/ml). The solution was then loaded into a 5ml polyallomer tube (Sorvall) and placed in a TV865 vertical rotor and centrifuged at 40,000 rpm for 18 hr at 20°C. The plasmid band was visualised using U.V. light (300nm) and collected using a 1ml hand held pipettman (Gilsen). The ethidium bromide was removed by extraction with butan-2-ol saturated with

diw. The DNA was precipitated with 2 volumes of 70% ethanol (-70°C for 30 min) and collected by a centrifugation for 15 min at 10,000 rpm. The DNA was resuspended in 1ml TE and then reprecipitated with 1/10 volume 3M sodium acetate (pH4.8) and 2.5 volumes of 100% ethanol. After further centrifugation the DNA was dried and resuspended in 500ul TE and the DNA concentration checked by agarose gel electrophoresis.

2.5 Restriction Endonuclease Digestion

Restriction enzymes were purchased from Boehringer-Mannheim, New England Biolabs, BRL or Amersham International. Digestions (usually containing 1-10ug plasmid DNA) were carried out in either low (10mM MgCl₂, 10mM Tris-HCL, pH7.5), medium (50mM NaCl, 10mM MgCl₂, 10mM Tris-HCl, pH7.5) or high salt (100mM NaCl, 10mM MgCl₂, 500mM Tris-HCl, pH7.5) buffers according to the manufacturers recommendations. Incubations times ranged between 2 and 12 hr at enzyme volumes up to 1/10 the total digestion volume. Reactions were stopped using 1/10 volume of STOP (20% Ficoll, 10mM EDTA).

2.6 Gel Electrophoresis of DNA

DNA samples were analysed using horizontal agarose gel kits (Sigma type II). Agarose was used at a concentration of 1% and the gels electrophoresed at a

voltage of 60V/gel in 1 x TBE buffer [89mM Tris-Borate (BDH), 89mM Boric acid (BDH), 2mM EDTA (BDH)]. Gels were then stained by the addition of 40µl of ethidium bromide (10mg/ml) to 1 x TBE buffer for 10 min. After de-staining the gels (10 min in 1 x TBE) DNA was visualised by UV illumination. Gels were photographed using Kodak film. The DNA size markers used during agarose gel electrophoresis were Hind III digested CI857 sam7 lambda phage DNA and HaeIII cut ϕ x174 coliphage DNA. Usually 1µg of marker DNA was loaded per track.

Molecular Marker	Lambda (<u>HindIII</u>)	ϕ x174 (<u>HaeIII</u>)
fragment size (bp)	23130 (cohesive)	1353
	9416	1070
	6682	1070
	4361 (cohesive)	872
	6682	603
	2027	310
	564	281
	125	271
		234
		194
		118
		72

2.7 Southern Blotting

DNA separated on agarose gels was transferred to nitro-cellulose or Hybond-N-nylon sheets (Amersham) according to Southern (1975). Gels were soaked for 45 min in denaturing solution (1.5M NaCl, 0.5M NaOH) under shaking conditions then transferred to neutralising solution (3M NaCl, 0.5M Tris-HCl, pH7.5) for a further 45 min. DNA was then transferred overnight from gel to Nitro-cellulose or Hybond in 20xSSC (3M NaCl, 0.3M sodium citrate) through a wick of 3mm filter paper. Transferred DNA was immobilised on the nitro-cellulose by baking at 80°C in vacuo for 1 - 2 hr or on Hybond by exposure to U.V. for 5 min.

2.8 Isolation of cDNA

cDNA inserts were isolated from the parental vector DNA by preparative gel electrophoresis and subsequent centrifugation through a 20µm spin-X columns (Costar). Plasmid DNA (10 - 20µg) was digested to completion with the appropriate restriction enzyme and DNA fragments separated by electrophoresis in a 1% agarose gel. Agarose containing the DNA band of interest was visualised using ethidium bromide staining, and excised using a scalpel. The agarose was finely chopped and placed in a spin-X column which was centrifuged at 13,500 rpm in a microfuge pre-chilled to 4°C. The DNA in the

resulting supernatant was precipitated by the addition of 1/10 volume 3M sodium acetate (pH4.8) and 2.5 volumes 100% ethanol. After incubation at -20°C overnight the DNA was collected by centrifugation, dried and resuspended in 10 - 20µl TE for further use.

2.9 Ligation of DNA Fragments

Ligations were carried out using 100 - 200µg of linearised vector DNA and a 5-fold molar excess of insert DNA in ligation buffer (50mM Tris-HCl pH7.5, 10mM MgCl₂) supplemented with 1mM DTT, 1mM rATP and 10 units of T4 DNA ligase in a total volume of 10 - 20µl. Incubations were carried out overnight at room temperature for cohesive end ligations or at 4°C for blunt end ligations.

2.9.1 Dephosphorylation of DNA Using Bacterial Alkaline Phosphatase (BAP)

EcoRI digested DNA (5µg) was dissolved in 1xCIP buffer (50mM Tris-HCl, pH9.0, 1mM MgCl₂, 0.1mM ZnCl₂, 1mM spermidine), to this was added 1 unit of BAP (Amersham) and the solution incubated at 37°C for 30 min. Another 1 unit of BAP was then added and the incubation continued for a further 30 min. To this was then added 1/10 volume of EDTA (0.2M) and SDS to a final concentration of 0.5%. The solution was then placed at 70°C for 10 min, then the volume made up to 50µl with TE buffer. The DNA was then

extracted with phenol, phenol/chloroform, chloroform and ether. Sodium acetate (3M) pH5.5 was added to 1/10 of the volume along with 2.5 volumes of 100% ethanol. The solution was incubated at -70°C for 60 min and the DNA recovered by centrifugation, desiccated then resuspended in TE buffer at a concentration of 0.2µg/ml.

2.10 Radiolabelling of DNA

2.10.1 Random Priming

The random priming of isolated cDNAs with radiolabelled ^{32}P dCTP was carried out as follows:-

diw to	30µl
OLB	3µl
BSA (10mg/ml)	1.2µl
DNA	25ng
^{32}P dCTP	30µCi
Klenow	1.2µl

Where OLB is a mixture of the following solutions (A,B and C) in a ratio of 2:5:3:

Solution A: 625µl 2M Tris HCl, pH8.0

25µl 5M MgCl₂

350µl diw

19µl 2-mercaptoethanol

5µl dATP in 3mM Tris-HCl, pH8.0, 0.2mM
EDTA pH7.0

5µl dTTP in 3mM Tris-HCl, pH5.0. 0.2mM
EDTA pH7.0

5µl dGTP in 3mM Tris-HCl, pH8.0, 0.2mM
EDTA pH7.0

Solution B: 2M Hepes titrated to pH6.6 with 5M NaOH.

Solution C: Hexadeoxyribonucleotides (Pharmacia) in
3mM Tris-HCl, pH8.0, 0.2mM EDTA, pH7.0,
to a concentration of 90 OD units/ml.

The random prime mix was incubated at 37°C for 2 hr. The 400µl of TE was added before boiling for 10 min to separate the DNA strands. The mix was then incubated for 15 min on ice prior to use.

2.10.2 Radiolabelling of Synthetic Linkers

Linkers were labelled using the polynucleotide kinase reaction as described by Manniatis et al (1982). DNA(2µg) was suspended in 6µl of TE to which was added 1µl of 10x Linker-kinase buffer [0.7M Tris-HCl, pH7.6,

0.1M MgCl_2 , 50mM DTT (Sigma)], 20 μCi ^{32}P dATP (Amersham) and 10 units of polynucleotide kinase (Amersham). This was then incubated at 37°C for 15 min whereafter a further 1 μl of 10x Linker-kinase buffer was added together with 10 units of polynucleotide kinase and 1 μl ATP (10mM) and the volume made up to 20 μl with diw. This solution was incubated for a further 30 min at 37°C. The linkers were then stored at -20°C until used.

Prior to use, linkers were tested for their ability to ligate as follows:- kinased linkers (100 μg), ligation buffer (50mM Tris-HCl, pH7.5, 10mM MgCl_2) T4 DNA Ligase (10 units) were incubated together for 4 hr at 4°C. The ligase was then heat inactivated at 65°C for 15 min. This solution was then analysed by electrophoresis through an 8% polyacrylamide gel run in 0.5xTBE. Also tested were kinased linkers which had not been ligated. After electrophoresis the gel was dried down, wrapped in Saran wrap and subjected to autoradiography at -70°C.

2.10.3 Klenow Reaction to Fill In the Cohesive Ends of DNA

The method used to remove cohesive was essentially that of Maniatis et al (1982). DNA (10 μg) was incubated in 1xnick-translation buffer (50mM Tris-HCl, pH7.2, 0.01M MgSO_4 , 0.1mM DTT, 50 $\mu\text{g}/\text{ml}$ BSA) containing 2nmols of

unlabelled dCTP, dGTP and dTTP. ^{32}P dATP (1 μCi) and Klenow enzyme (2 units) (Amersham) was then added. The solution was incubated at room temperature for 10 min, then 2 nmols of unlabelled dATP was added and the reaction continued for a further 20 min. The Klenow enzyme was then heat inactivated at 70°C for 5 min. 1/10 volume of the solution was loaded onto a 1% agarose gel then after electrophoresis the gel was dried down and subjected to autoradiography to check the incorporation of ^{32}P dATP as a measure of the success of the reaction.

2.11 Hybridisation of Radiolabelled cDNA To Nucleic Acids Bound To Membranes

Filters were prehybridised at 68°C for 45 min in sealed plastic bags.

Prehybridisation Solution:

- 10% Dextran sulphate
- 5x SSC
- 0.1% pyrophosphate
- 0.5% SDS
- 150-200ug/ml denatured salmon sperm DNA
- 2x Denhardtts

where 2 x Denhardtts:

- 0.02% BSA (Sigma)
- 0.02% PVP 360 (Sigma)
- 0.02% Ficoll 400 (Sigma)

Radiolabelled cDNA probe was then added to the bag and hybridised overnight at 68°C. Filters were washed for 4 x 30 min in hybridisation wash (2x SSC, 0.1% SDS and 0.1% pyrophosphate) at 68°C.

2.12 Autoradiography

Radioactive ^{32}P -labelled nucleic acid probes hybridised to nitro-cellulose or Hybond filters were visualised by exposure of Kodak X-ARS film. Filters were placed at -70°C (for 1 - 7 days) in X-ray cassettes with intensifying screens with the film placed on top. Films were developed using a Gevomatic 60 (AgFa-Gevaert) automatic developer.

2.13 RNA Analysis

2.13.1 RNA Extraction Using Guanidinium Hydrochloride

RNA was extracted from yeast cells using a variation of the method of Cox (1968) was used. Yeast cultures were grown to late logarithmic phase (optical density at 600nm = 0.6) then pelleted by centrifugation at 3,000 rpm for 10 min. Spheroplasts were then prepared as described in Section 2.16.2, pelleted and following resuspension in 10ml of 8M guanidinium hydrochloride, disrupted using a hand-held homogeniser. Cell debris was removed by centrifugation at 7,000 rpm and the supernatant transferred to a sterile tube. 100% ethanol

(0.5 volumes) was then added prior to precipitate the RNA (45 min incubation at -20°C). The precipitate was collected by centrifugation at 10,000 rpm for 15 min. The pellet was resuspended in 5ml of 6M guanidinium hydrochloride and 0.5 volumes of cold 100% ethanol prior to a further incubation at -20°C for 45 min. The precipitate was pelleted as before and the 6M guanidinium hydrochloride treatment repeated. The resultant pellet was resuspended in sterile DEPC-treated diw (2ml) and 1/10 volume 2M sodium acetate (pH5.5) added along with 2.5 volumes cold 100% ethanol. Samples were incubated at -20°C overnight. RNA was pelleted by centrifugation at 10,000 rpm for 15 min and resuspended in 1ml of sterile DEPC-treated diw. The RNA concentration was determined by measuring the optical density at 260nm (for RNA OD at 260nm = 1 = 40 $\mu\text{g}/\text{ml}$) and stored at -70°C .

2.13.2 Gel Electrophoresis of RNA

RNA was size fractionated through formaldehyde denaturing gels by a modification of Derman et al (1981) according to Meehan et al (1984). Samples were suspended in 50% formamide, 6% formaldehyde and 10mM sodium phosphate, pH6.5 and samples were heated to 50°C for 15 - 30 min prior to loading. Bromophenol blue/orange G-dye was then added in the presence of 10% Ficoll and the samples were separated at 30V for 16-30 hr in 10mM

sodium phosphate buffer pH6.5. Gels were then soaked in 10xSSC prior to transfer to nitro-cellulose. Transfer was carried out overnight in 10xSSC according to Southern (1975). Filters were baked in vacuo at 80°C for 1 - 2 hr to bind the RNA.

2.14 Microbial Transformation With Plasmid DNA

2.14.1 Bacterial Transformation Using Calcium Chloride

Competent E.coli cells were prepared using the calcium chloride method as described by Maniatis et al (1982). The strain of interest was grown overnight in L-broth then diluted (1/100 v/v) into fresh media (50ml). The culture was grown at 37°C until the absorbance at 550nm = 0.6 then chilled on ice for 20 min. Cells were collected by centrifugation and resuspended in 50mM CaCl₂ (10ml). After a further 20 min incubation on ice, cells were sedimented and again resuspended in 50mM CaCl₂ (5ml). Cells were stored at 4°C and used within 4 days after preparation.

Transformation was carried out as follows:-

DNA (10ng - 1µg) was added to 200µl of competent cells and left on ice for 30 min and then heat-shocked at 42°C for 90 secs. L-broth (500µl) was then added and the culture placed at 37°C for 60 min to allow the expression of the selectable marker. This culture (200µl) was then plated onto L-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.14.2 Yeast Transformation Using Lithium Acetate

Competent S.cerevisiae were prepared using lithium acetate as described by Itoh et al (1983). The strain of interest was grown overnight in YPDA to stationary phase then diluted (1/100 v/v) into YPDA (50ml). The culture was grown until the optical density at 600nm reached 0.4 then harvested by centrifugation. The pellet was washed in 10ml TE and sedimented again. After resuspending the pellet in 5ml of TE, lithium acetate (Sigma) was then added to a final concentration of 0.1M and the cells incubated at 30°C for 60 min with gentle shaking. DNA (10µg) was then added to 200ul aliquots of cells and the mixture incubated at 30°C for a further 30 min. PEG-4000 (Sigma) was added to a final concentration of 35% (v/v) and the mixture incubated for 60 min at 30°C. The cells were then heat-shocked at 42°C for 5 min. Cells were pelleted and resuspended in sterile diw (1ml). Following further sedimentation the cells were resuspended in 100ul sterile diw and then plated onto yeast minimal agar plates with the appropriate selection.

2.15 Screening of Bacterial Colonies for Recombinant Vectors

The method of Grunstein & Hogness (1975) was used to bind DNA from plasmid-containing bacterial colonies to nitro-cellulose. An array of colonies were streaked onto

duplicate nitro-cellulose sheets (Schleicher & Schuell) placed onto L-agar plates containing ampicillin (50µg/ml). Filters were marked for orientation and the plates incubated at 37°C overnight. The masterplate was then stored at 4°C. Filters to be probed were placed colony side up for 3 min on a sheet of Whatman 3mm chromatography paper soaked in 10% SDS. Filters were then transferred to Whatman paper soaked in denaturing solution (0.5M NaCl, 1.5M NaOH) for 5 min then to paper soaked in neutralising solution (0.5M Tris-HCl, pH7.5, 1.5M NaCl) for a further 5 min. Filters were then soaked in 2xSSC for at least 5 min, air-dried at room temperature and baked in vacuo at 80°C for 90 min to bind the DNA to the nitro-cellulose.

Filters were then hybridised to radiolabelled DNA probes and positive colonies obtained from the master plate. Plasmid preparations were then carried out on these colonies.

2.16 Preparation of Yeast Subcellular Fractions

2.16.1 French Press Method

Late logarithmic phase yeast cultures (0.5 - 2l) grown in synthetic SD medium supplemented with casamino acids (1%) were harvested by centrifugation at 4°C (all subsequent steps were also carried out at 4°C). Cells were resuspended in microsome buffer (10ml) (0.1M



potassium phosphate pH7.4, 10mM EDTA, 0.25mM PMSF, 20% glycerol) if microsomes were required. If cytosols were required cells were suspended in KCl phosphate buffer (0.1M potassium phosphate, pH7.7, 0.1mM EDTA, 165mM KCl) Cell suspensions were then passed through a French Press and the cell debris removed by centrifugation at 8,000 rpm for 10 min. The supernatant was then respun for 20 min at 8,000 rpm to remove the mitochondrial fraction. The supernatant was then transferred to 6.5ml polycarbonate tubes (Sorvall) centrifuged at 45,000 rpm for 90 min in a Sorvall TFT45.6 rotor to separate the cytosolic (supernatant) and microsomal (pellet) fractions. Microsomal fractions were resuspended in microsome buffer using a hand-held teflon/glass homogeniser (avoiding cavitation). Cytosolic and microsomal fractions were aliquoted in 1ml volumes and either used directly for further biochemical analysis or stored at -40°C until required.

2.16.2 Lyticase Spheroplast Preparation

Late logarithmic yeast cultures (11) were harvested at 4°C (5,000 rpm, 10 min) then resuspended in 20 ml of reagent 1 (0.1M potassium phosphate, pH7.2, 0.1M EDTA, 0.1M 2-mercaptoethanol) and incubated for 30 min at room temperature. Cells were collected and washed twice in ice-cold diw and twice in reagent 2 (0.1M potassium

phosphate, pH7.2). The wet cell weight was determined and the cells resuspended in 50ml of reagent 3 (0.1M potassium phosphate, pH7.2, 10mM EDTA, 0.9M sorbitol (Sigma) containing 2.5mg lyticase (Sigma) per gram wet weight of cells. This solution was incubated at 37°C for 60 min in a 1 litre conical flask with gentle shaking (100 rpm). The cells were then pelleted (1,000 rpm, 10 min) at 4°C and the supernatant carefully discarded. The spheroplasts (and undigested cells) were then resuspended in 10ml of ice-cold microsome buffer and disrupted in a hand-held teflon/glass homogeniser. Microsomal and cytosolic fractions were then prepared as in Section 2.16.1.

2.16.3 Sonication Method

Late logarithmic or stationary phase yeast cultures (50 - 100ml) were harvested by centrifugation (3,000 rpm, 5 min). Cells were resuspended in 2ml of KCL phosphate or microsome buffer and subjected to six 15 second sonications using a Soniprep 150 sonicator (MSE) at full power. Each cell suspension was held on ice for 2 min between steps. Cell debris was then removed by centrifugation (3,000 rpm, 10 min) and the supernatant used for further biochemical analysis.

2.17 Biochemical Assays

2.17.1 Protein Estimation

The protein content of yeast samples was determined using the method of Lowry et al (1951) using Bovine serum albumin (BSA, Sigma) as a standard. The reagents used were as follows:-

1. 70mM sodium carbonate:40mM NaOH
2. 40mM copper sulphate
3. 71mM sodium potassium tartrate
4. folin Coicalteau reagent (diluted 1:2 with diw)
4. 0.1M NaOH
6. Freshly prepared alkaline copper solution (49ml reagent 1, 0.5ml reagent 2, 0.5ml reagent 3).

Samples were diluted to a volume of 1ml with 0.1M NaOH to give a protein concentration within the standard range (0-200 μ g). Reagent 6 (5ml), was then added and after a 10 min incubation at room temperature 500 μ l of reagent 4 added. The solution was incubated for a further 30 min at room temperature and the absorbance at 600nm was determined using a Shimadzu U.V. 160 spectrophotometer. The protein concentration was calculated from a standard curve using BSA (0;25,80;100;120;150;200 μ g/ml). The absorbance of the standards was determined in duplicate and that of the samples in triplicate.

In some cases the protein concentration was very low, for example, when 50ml yeast cultures were used. In these cases a micro Lowry was used where all the sample and reagent volumes were decreased by a factor of 5.

2.17.2 Measurement of Cytochrome P-450 Haemoprotein

This was carried out according to the method of Omura & Sato (1964). Yeast cultures (50ml) were grown in synthetic SD medium supplemented with 1% casamino acids. Cells were harvested in late logarithmic phase (Optical density at 600nm = 0.7), washed once in ice-cold diw and resuspended in 2ml of 0.1M potassium phosphate, pH7.4. Cell suspension (1ml) was added to both sample and reference cuvettes and a few milligrams of sodium dithionite added to each to reduce the cytochrome P-450. Carbon monoxide was then bubbled through the sample curvette and the reduced carbon monoxide difference spectra recorded at room temperature between 500 and 400nm using a Shimadzu MPS-2000 scanning spectrophotometer. Cytochrome P-450 haemoprotein concentration was determined using an extinction coefficient (450 - 490nm) of $91\text{mM}^{-1}\text{ cm}^{-1}$.

2.17.3 Cytochrome c Reductase Assay

1ml of cytochrome c (Sigma) (1.5 mg/ml) was dissolved in 0.1M potassium phosphate buffer, pH7.4 and

heated to 37°C in a spectrophotometer cuvette. Yeast cellular fractions (10µl) were added to the cytochrome c solution. The absorbance of each sample was then measured at 550nm against a reference cuvette (containing only cytochrome c solution) on a Shimadzu MPS-2000 scanning spectrophotometer to obtain a base-line. NADPH was then added to the sample cuvette (final concentration of 160µg/ml). The subsequent reduction of cytochrome c was then measured at 550nm for at least 2 min. This was repeated at least three times for each sample.

2.17.4 Cytochrome P-450 Mediated Metabolism of 7-Benzoyloxyresorufin

The metabolism of benzyloxyresorufin to resorufin was measured flurometrically using a modified version of that described by Wolf et al (1986). Yeast microsomal fraction (5 - 20mg protein) was added to 1ml of 1.0µM 7-benzyloxyresorufin in 0.1M Tris-HCl, pH7.4. The sample was equilibrated at 37°C then 5µl of NADPH (8mg/ml) added. The change in fluorescence (at excitation 530nm and emission 585nm) was then measured for at least 3 min before adding 5µl of resorufin standard (10µM). At least three determinations were made for each sample.

2.17.5 Glutathione S-transferase Activity Towards 1-Chloro-2,4-dinitrobenzene (CDNB)

The activity towards CDNB was determined by the method of Habig *et al* (1974) by measuring the loss of spectral absorption at 340nm at 37°C. Assay buffer was prepared by dissolving 41mg of CDNB (Sigma) in 4ml of 100% ethanol and adding this to 196ml of 0.1M potassium phosphate buffer, pH6.5 warmed to 55°C. Assays were carried out in 1cm quartz cuvettes using a Shimadzu MPS 2000 scanning spectrophotometer, 20mM GSH (50ul) was added to the sample cuvette and the background (non-enzymic) rate of GSH conjugation measured at 340nm. For each assay 5 - 50ul of yeast soluble fraction (1mg protein) was added to the sample cuvette and the enzymatic rate of GSH conjugation measured. Samples were tested in duplicate.

2.17.6 Glutathione Peroxidase (GPX) Measurements

2.17.6A Total GPX Activity Using Cumene Hydroperoxide

Total GPX activity was measured using the method of Paglia & Valentin (1967) at 37°C using Cumene hydroperoxide (CHP) as substrate. Assay buffer (100mM Tris-HCl, pH7.2, 3mM EDTA, 1mM sodium azide (Sigma), 1.2mM CHP, 0.5mM NADPH, 1 unit glutathione reductase (Sigma)) in a final volume of 1ml was used for each assay. Cytosolic samples (5 - 10mg protein) were then added and

the rate of NADPH oxidation monitored at 340nm on a Shimadzu MPS 2000 scanning spectrophotometer.

2.17.6B Selenium Dependent GPX Activity

This assay was carried out as for total GPX activity using hydrogen peroxide (0.25mM) (BDH) instead of the 1.2mM CHP in the assay buffer.

2.17.6C Non-Selenium Dependent GPX Activity

The non-selenium GPX activity was calculated by subtracting the value for the selenium-dependent GPX activity from the total GPX activity (Masukawa et al, 1983). This activity gives an estimate of the contribution of the alpha class GST to the CHP activity measured.

2.17.7 Measurement of Glutathione (GSH) Levels Using Ortho-phthaldehyde (OPT)

The method used to estimate yeast cellular GSH levels was that of Hissin & Hilf (1976). Yeast soluble fractions (50µl) were mixed 1:1 with 10% trichloroacetic acid to precipitate protein and, by acidifying the sample, prevent GSH auto-oxidation. Duplicate aliquots of each sample were added to 1.8ml of GSH assay buffer (0.1M sodium phosphate buffer, pH8.0, 5mM EDTA). Freshly prepared OPT (1mg/ml) (Sigma) in methanol was then added

and the mixture incubated at room temperature in the dark (15 min). The fluorescence of the GSH-OPT conjugate was determined using a Perkin-Elmer fluorescence spectrophotometer (model LS-3, excitation 350nm and emission 420nm). GSH levels were calculated from a standard curve obtained using GSH standards ranging between 0.05 - 5uM.

2.18 SDS-Polyacrylamide Gel Electrophoresis(SDS-page)

SDS-page was carried out using the method of Laemmli (1970). Glass plates (12 x 14cm) and spacers were washed with 100% ethanol then diw and dried thoroughly then assembled into a gel sandwich in the protean I gel apparatus (Biorad). Either 12% or 9% polyacrylamide separating gels were used depending on whether GST(12%) or cytochrome P-450(9%) samples were analysed. These were prepared as follows:-

polyacrylamide

concentration	acrylamide	SGB	diw	1% APS	TEMED
	(ml)	(ml)	(ml)	(ml)	(μl)
9%	11.1	9.25	14	2	20
12%	14.8	9.25	11.0	2	20

where SGB is separating gel buffer (1.5M Tris, 0.5% SDS, pH8.0)

1% APS = ammonium persulphate

TEMED (Sigma) = N,N,N¹,N¹-tetramethylethylenediamine

The acrylamide buffer and diw then the 1% APS and TEMED added. The gel was poured to a height of 12cm then carefully overlaid with diw and allowed 45 min to set. Prior to preparing the stacking gel the diw overlay was removed and a 15 or 20 track comb (washed with 100% ethanol and diw) inserted. The 4.5% polyacrylamide stacking gel was prepared using 1.5ml of 30% acrylamide, 2.5ml stacking gel buffer (0.5M Tris-HCl, 0.5% SDS, pH6.8) 5.7ml diw, 0.3ml 1% APS and 10µl TEMED. After mixing, this solution was poured onto the separating gel and left for 45min to set. The comb was then removed and the wells washed with electrode buffer (0.052M Tris, 0.053M glycine, 0.1% SDS, pH8.3). The gel was then coupled to the upper reservoir of the Protean apparatus and placed in the lower reservoir to which had been added 1 litre of electrode buffer.

The electrodes in the upper reservoir were then submerged using the electrode buffer. Samples (25-100µg) were diluted 1:1 with boiling mix (10% stacking gel buffer, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and heated to 100°C for 5 min. Samples (25-100ug) were loaded using a Hamilton syringe (Hamilton Bonaduz). The gel was run at a current of 20mA/gel while the dye front was in the stacking gel and

at 30mA/gel through the separating gel. Electrophoresis was continued until the dye front was within 1cm of the bottom of the gel.

2.19 Western Blot Analysis

Western blots were carried out according to the method of Towbin et al (1979) as modified by Lewis et al (1988). Following SDS-page the stacking gel was removed and the separating gel cut along the dye front. The gel was then marked for orientation (usually by cutting the top right hand corner) and loaded into a transblot cassette (Biorad) according to the manufacturers instructions. The cassette was then placed in a transblot tank (Biorad) containing transblot buffer (20mM disodium orthophosphate, 20% methanol) with the nitro-cellulose adjacent to the anode. Protein transfer was then carried out overnight at 250mA. The cassette was then opened and the nitro-cellulose cut using the gel as a template and marked for orientation. The nitro-cellulose was washed for two 10 min periods in TBST (50mM Tris-HCl pH7.9, 0.15M NaCl, 0.05% Tween 20 (Sigma)) then blocked for 60 min in 3% Marvel (Cadburys). Two 10 min washes were then carried out prior to a 60 min incubation with antibodies raised against either the P450IIB1 and P450IIC1 proteins or the alpha or pi class

GST subunits. All antisera were diluted 1:500 in TBST. Following three 10 min washes in TBST the nitro-cellulose was incubated for 60 min with 1:1000 diluted donkey antirabbit antibody conjugated to horseradish peroxidase (Scottish Antibody Product Unit) in TBST. Following a further three 10 min washes in TBST the protein bands were visualised using:

a) Peroxidase staining with 4-chloro-1-naphthol (Sigma) as substrate. Nitro-cellulose filters were placed in 200ml of TBS (50mM Tris-HCl, pH7.9, 0.5M NaCl) and 4-chloro-1-naphthol (120mg) dissolved in 40ml of methanol. To this hydrogen peroxide (30%, 80 μ l) was then added. When bands were visible the solution was discarded and replaced by diw.

The filters were then labelled with ^{125}I protein A as below.

b) ^{125}I protein A labelling. Filters were placed in 50ml of TBST containing 0.19MBq ^{125}I protein A (Amersham) for 45 min. Non-hybridised ^{125}I protein A was removed by washing the nitro-cellulose in TBST. Filters were then dried between 3mm Whatman paper. The nitro-cellulose was then transferred to fresh Whatman, covered in Saran wrap, and subjected to autoradiography.

2.20 L-Canavanine Mutation Assay

2.20.1 Isolation of CAN1 Colonies

This was carried out using a modified version of Lemontt (1977) where CAN1 colonies were selected from KY118 containing p56/3a (P450IIB1 expressing) or p56 par (control). This was achieved by replica plating colonies grown on supplemented synthetic SD agar [in the absence of L-tryptophan (Sigma)] onto agar containing 30µg/ml L-canavanine (BDH). Those colonies that showed no growth when exposed to the L-canavanine were designated CAN1, i.e. had a functional arginine permease and were selected for use in subsequent mutation assays.

2.20.2 Use of CAN1 Strains In Mutation Assay

Single CAN1 colonies of strain 56/3a or 56 par were inoculated into supplemented synthetic SD medium containing 1% casamino acids and grown into late logarithmic phase. Cultures were then counted using a haemocytometer (Neubauer) and 4×10^7 cells transferred to a 30ml sterile Universal tube (sterilin). Cells were pelleted at 4°C then resuspended in 1.96ml of fresh medium. They were subsequently treated with the desired chemical at a variety of concentrations. Chemicals were dissolved in 40µl DMSO (BDH). Control cultures contained only 40µl DMSO. Cultures were treated for 2 hr at 37°C in an orbital shaker. The temperature was lowered to

30°C for a further 16 hr to allow the expression of the canavanine resistance phenotype (can1). Cultures were counted and 1×10^7 cells were plated onto supplemented minimal agar plates containing 40µg/ml L-canavanine. Cultures diluted to give 200 cells were also plated onto YPDA agar plates to determine cell survival. Plates were incubated at 28°C for 3 days to determine survival and 6 days to determine canavanine resistance. Plates were

scored and mutations/ 10^6 survivors calculated. Results were then normalized by dividing by the background mutation frequency. Thus the plotted mutation frequency was $\frac{\text{mutations}/10^6 \text{ survivors}}{\text{background}/10^6 \text{ survivors}}$

2.20.3 Nitrous Acid Mutation Assay

Nitrous acid (0.2g/ml) was prepared and added to log phase yeast cells (2×10^7 /ml) carrying either p56/3a or p56 par to give a concentration of 1 or 2 mg/ml nitrous acid. Each culture was kept at pH4.5 using the appropriate pH buffer tablets (BDH). The reaction was carried out at 30°C in an orbital shaker for 15 min. The exposure was terminated by increasing the pH to 7.0. Cells were then diluted 10,000 fold and 100µl aliquots plated onto selective (L-canavanine containing) plates and YPDA agar plates to score mutation and survival respectively.

2.21 Yeast Cytotoxicity Assays

Overnight cultures of yeast strains carrying the appropriate plasmid were inoculated into fresh supplemented synthetic SD medium at a concentration of 2×10^5 cells/ml. After an overnight incubation at 28°C in an orbital shaker (200 rpm) culture densities were measured using a haemocytometer. Cells (4×10^7) were removed to a sterile Universal tube, pelleted at 4°C, and then resuspended in 1.96ml of PBS (oxoid). Test chemical was then added in 40µl of the appropriate solvent. Cells were incubated for 2 hr at 37°C with vigorous aeration, diluted 10,000 fold and then 100µl aliquots plated in triplicate onto YPDA agar plates. Plates were incubated at 28°C for 3 days then scored.

For each culture tested the CDNB activity at the time of drug addition was determined.

2.22 Cytotoxicity Assay After Depletion of Yeast Cellular GSH Levels

GSH levels in the yeast strains were depleted using the γ -glutamyl cysteinyl synthetase inhibitor, buthionine-S,R-sulphoximine (BSO) (Sigma). BSO was prepared in diw which was filter sterilised then added to overnight cultures at a final concentration of 10mM. The GSH levels were estimated either in the presence or absence of BSO using the OPT assay (Section 2.17.7) then treated as in Section 2.21.

CHAPTER 3

EXPRESSION OF RAT CYTOCHROME P450IIB1 IN S.CEREVISIAE

3.1 Introduction

Current concerns over environmental issues exemplifies the need for rapid methods to assess the health risk when humans are exposed to drugs and chemicals. The DNA damage resulting from exposure to environmental mutagens (both natural and man-made) is likely to be a major cause of cancer (Doll, 1977). This exposure occurs from natural chemicals in the diet as well as from accidental or deliberate exposure to synthetic chemicals or complex mixtures such as cigarette smoke. Chemical-induced mutation in germ cells can result in genetic defects which may appear in future generations. At the somatic cell level cancerous cells may result from modifications in the cell processes that would normally control cell proliferation.

The available data supports the hypothesis that environmental factors play a major role in cancer, but how is exposure to be correlated with cancer incidence? For example, in Japan there is a very low incidence of breast and colon cancer but a high level of stomach cancer. In the USA the reverse is found to be true. However, Japanese who emigrate to the USA will, within a generation or two, show high levels of breast and colon cancer while having the low stomach cancer incidence of native americans (Ames, 1979). So how are the factors involved in this cancer incidence to be unravelled?

One way of linking xenobiotic exposure with cancer is to use epidemiology. Unfortunately humans typically have a 20 - 30 year latency period between the initial xenobiotic exposure and cancer appearance. For example, men first smoked cigarettes in great numbers around 1900, but the resultant increase in lung cancer did not become apparent until 1925 - 1930 (Ames, 1979). Thus human epidemiology cannot be used as a front-line method in the determination of individual carcinogens because of the long latency period involved.

Animal cancer tests are another method for screening potential carcinogens, however, these studies again have severe limitations. There are over 50,000 synthetic chemicals in widespread use and this number is increasing at the rate of 1000 new compounds per year (Lowe & Omenn, 1986). Only a small percentage of these chemicals were actually screened for their potential carcinogenicity or mutagenicity prior to introduction. Also only 150 chemicals per year (in 1979) were actually screened using animal tests. So questions arise as to the ability of animal tests to keep pace with the development of new chemicals. The tests themselves are also prohibitively expensive (> \$1 million, in 1986), take too long (2 - 4 years) to allow drug and chemical companies to test and remove unsatisfactory chemicals while still in development (Lowe & Omenn, 1986). The sensitivity of

animal cancer tests is also questioned. If an environmental carcinogen was to affect only 1% of 100 million people then this would result in one million cases of cancer. But to detect a chemical causing cancer in 1% of test animals would require 10,000 animals which is obviously unfeasible. This limitation is overcome, somewhat, by exposing animals to their maximum tolerated doses. This increases the tumour incidence and reduces the problems associated with population size but does this reflect the risk linked with human exposure?

Short-term tests (STT) have thus been introduced to screen potentially mutagenic chemicals. These have been developed from initial experiments designed to study the mechanisms of DNA damage induced by chemical treatment. Their role has increased due to reports that rodent carcinogens are mutagenic in STT (Ames et al, 1973). These tests have advantages because they are quick and cheap compared to epidemiological or animal studies. Originally sensitivities (percentage carcinogens identified as mutagens) and specificities (percentage non-carcinogens identified as non-mutagens) of greater than 90% were reported (Purchase et al, 1976).

In 1984 the National Toxicology Program (NTP) initiated a study to assess the four most commonly used in vitro STT. These were the Ames test using S.typhimurium, chromosome aberration and sister chromatid exchange in chinese

hamster ovary cells and the mouse lymphoma L5178Y cell mutagenesis assay (Tennant et al, 1987). It was found that testing 73 chemicals using standard protocols for each assay gave only 60% concordance between carcinogenicity and mutagenicity (as compared to the claims of >90%). Thus no single in vitro STT adequately predicts the diverse mechanisms involved in carcinogenesis. Furthermore there was shown to be no advantage to be gained in using a battery of STT (Tennant et al, 1987).

The problem is that the present STT have the significant limitation in that they mostly rely on the exogenous application of drug metabolising enzymes. These enzymes in the form of mammalian subcellular fractions convert the test chemical to its toxic or mutagenic products (Lu & West, 1986; Wolf, 1986; de Montellano, 1986). The subcellular fractions used invariably contain mammalian cytochrome P-450 (P-450). P-450 metabolism, though generally associated with the detoxification and excretion of lipophilic compounds, can also generate chemically reactive nucleophilic products. It is as a consequence of such reactions that a vast range of chemicals are mutagenic and carcinogenic (de Montellano, 1986). The commonly used STT are limited by poor correlation between mutagenic activity and carcinogenicity in the extrapolation of this data to man (Ashby, 1986; Tennant et al, 1987).

S.cerevisiae has been evaluated in the screening of compounds for mutagenicity (Callen & Philpot, 1978, Venitt & Parry, 1988). However, as previously mentioned endogenous yeast P-450's have narrow specificities being principally involved in ergosterol biosynthesis (the 14 α demethylase system) this being the major yeast membrane sterol (Yoshida & Aoyama, 1984). While their level is limited by the physiological state of fermentation (Wiseman & King, 1982). This restricts the use of this test system although, as in the Ames test, hepatic S9 fractions have been used to increase the sensitivity (Larimer et al, 1978). This also has the limitation in that the active metabolite is formed outside the cell and is required to pass through the cell wall and cytoplasm before any DNA interaction can occur. The expression of mammalian P-450 cDNA's in S.cerevisiae thus represents an attractive approach for the development of a new system. S.cerevisiae represents a suitable organism especially because high level protein production can be achieved and it is readily manipulated by standard molecular biological techniques.

The first successful expression of a mammalian P-450 cDNA was carried out in yeast using a multicopy yeast expression vector (pAAH5) with the constitutive alcohol dehydrogenase promoter, ADH1 (Oeda et al, 1985). Other expression systems have since been developed including

the monkey kidney COS cell expression system (Zuber et al, 1987). This transient system was used to express the bovine 17 α -hydroxylase cDNA. The protein levels that have been achieved, however, were extremely low. The vaccinia virus vector system has been used to express high levels of P-450 protein (Faletto et al, 1988) but this again is a transient system as the virus lyses the host cell. Recently cell lines have been developed which stably express P450IIB1 in V79 cells (Doehmer et al, 1988) which when exposed to the promutagen aflatoxin B₁ showed a four-fold increase in mutation rate, (Doehmer et al, 1988).

However, by far the most expression studies using P-450 have been carried out in S.cerevisiae. Oeda et al (1985) successfully expressed the rat P450IA1 cDNA in yeast to a level of 0.8% total yeast cellular protein. It was found that 50% of this protein contained heme and was both spectrally and enzymatically active (Oeda et al, 1985). This latter finding was important since it proved that a mammalian P-450 could couple efficiently with the endogenous yeast NADPH-dependant cytochrome P-450 reductase (reductase). As a consequence of these studies many P-450's have been expressed in yeast with a wide variety of protein levels achieved from very low to extremely high (Gonzalez, 1988). Other experiments have been carried out that show that although mammalian

P-450's will function with a yeast reductase the enzymatic activity is greater (around two-fold) if the mammalian reductase is expressed in the same cell (Murakami et al, 1986). This is of importance if S.cerevisiae is to be used as a host for P-450's either for structure/function studies or to develop the organism as an STT.

Other work has shown that a hybrid protein produced in yeast by fusing amino acid residue 57 of the rat P-450 reductase to the carboxy terminus of rat P450IA1 will give a protein that is catalytically self-sufficient (reductase independent). This hybrid has four times the activity of the rat P450IA1 protein expressed with only the yeast reductase in the cell (Murakami et al, 1987). Finally, P-450 expression in yeast facilitates site-directed mutagenesis (SDM) studies to study the role of specific amino acid residues around the heme binding cysteine in determining the spectral properties, and catalytic activity of the enzyme (Shimizu et al, 1988). Such studies have also been used to identify amino acid residues determining substrate specificity (Sakaki et al, 1987). Expression in yeast has also been used to examine the effect on catalytic activity of the point mutations found in a mouse P450IA1 cDNA isolated from a C37 hepatoma cell line (Kimura et al, 1987).

To date, however, there have been no studies in

which the rat P450IIB1 protein has been expressed in yeast. The suitability of a yeast strain expressing a mammalian P-450 as a mutation test organism has also not been examined. It was to this end that the rat P450IIB1 protein was expressed in the S.cerevisiae strain KY118 and its mutation rate determined on exposure to a range of chemicals. It was hoped to show the potential of a battery of yeast strains expressing mammalian activation systems. These could be used as STT to routinely screen new chemicals for mutagenic activity.

The rat enzyme studied, P450IIB1, is one of the two major phenobarbital (PB) inducible P-450's, the other being P450IIB2. These two proteins are immunochemically indistinguishable (Atchinson & Adesnik, 1986). At the amino acid level there are only 14 substitutions in 491 residues giving a 97% homology between the two proteins (Atchinson & Adesnik, 1986). They also have similar substrate specificities. However, the purified P450IIB1 protein is usually found to be the more active. It is five times more active in benzphetamine (Guegerich et al, 1982) or testosterone metabolism (Waxman et al, 1983). When benzo(a)pyrene or 7,12 dimethyl benz(a)anthracene are used as substrates P450IIB1 is seen to be 2-3-fold more active (Wilson et al, 1984). However, most interesting is the metabolism of certain resorufin analogues, for example pentoxyresorufin, where

there is a 100-fold greater rate of metabolism by P450IIB1 (Wolf et al, 1988).

There are other members of the rat P450IIB family which are at best marginally inducible by PB (Gonzalez, 1988). The data acquired to date suggests the presence of six genes in the rat (Gonzalez, 1988). There is thought to be considerable nucleotide similarity but whether all six are expressed is as yet unknown (Adesnik & Atchinson, 1986; Gonzalez, 1988). Members of the P450IIB subfamily have been isolated from several other species such as rabbit, man and mouse. Again there are thought to be multiple genes in each species.

Studies have shown that the mechanism of P-450 induction by PB requires both de novo RNA and protein synthesis (Adesnik & Atchinson, 1986). Enhanced levels of P450IIB2 mRNA could be detected within 3 hr of PB treatment which peaked at 16 hr then declined. The peak mRNA level was 25-100-fold higher than the level measured in untreated animals and accounted for approximately 1% total liver poly A⁺ mRNA (Adesnik et al, 1981). It was concluded that as protein levels increase concurrently with mRNA that PB regulation of P450IIB1 and P450IIB2 synthesis is primarily at the level of transcription (Adesnik & Atchison, 1986). Other chemicals are known to induce P450IIB1 these include barbiturates, mephentyoin, trans-stilbene oxide, xylene,

SKF-525A, chlordane, Kepone, isopropanol (weakly), 1,1-di(p-chlorophenyl)-2,2-dichloroethylene, allylisopropylacetamide and polyhalogenated biphenyls. Table 3.1 lists the catalytic activities which have been examined for P450IIB1 and for which significant activity has been measured. This table emphasises the broad substrate specificity of this enzyme.

RESULTS

3.2 Subcloning of P450IIB1 into pUC-9

The isolation and subcloning of the P450IIB1 cDNA used in these experiments has previously been described in detail (Doehmer *et al*, 1988; Monier *et al*, 1988). The full-length cDNA was assembled from portions of two overlapping P450IIB1 partial cDNA clones and a portion of a genomic clone for P450IIB2 providing the 5' coding sequence (the P450IIB2 sequence is identical to P450IIB1 over this region). The resultant full-length cDNA was sub-cloned into the expression vector pSP64 to give the plasmid, pSP-450. This was used by Adesnik and co-workers for *in vitro* transcription/translation experiments to study the N-terminal amino acid sequences of the protein involved in the cotranslational insertion of the protein into the endoplasmic reticulum (Monier *et al*, 1988).

Since the ultimate goal was the expression of the P450IIB1 cDNA from the yeast expression plasmid, pMA56, the restriction sites flanking the coding sequence of the

Activity
Acetanilide 4-hydroxylation
Acrylonitrile epoxidation
Aflatoxin B ₁ (covalent binding to DNA)
Aldrin epoxidation
Aminopyrine N-demethylase
Androstenedione
16 β -Hydroxylation
16 α Hydroxylation
Aniline 4-hydroxylation
Arachidonic acid
20-Hydroxylation
19-Hydroxylation
Epoxidation (all four positions)
Azoxypcarbazine N-oxidation
Benzyl
Methyl
Benzo(a)pyrene hydroxylation
α -Benzphetamine N-demethylase
3- <i>t</i> -Butyl-4-hydroxyanisole O-demethylation
1-(2-Chloroethyl)-3-(cyclohexyl)-1-nitrosourea
cis-3-Hydroxylation
cis-4-Hydroxylation
1-(2-Chloroethyl)-3-(<i>trans</i> -4-methyl cyclohexyl)-1-nitrosourea (several positions)
Cyclohexane hydroxylation
Cyclopropylamine and cyclopropyl ether oxidation
Dichlorinated biphenyl oxidation

Table 3.1 Catalytic Activities of the Rat P450IIB1 Protein.

The table details the catalytic activity of the rat P450IIB1 protein (P-450_{PB-B}) towards a variety of structurally diverse xenobiotics. Activity is expressed as nmol product formed/min/nmol P-450 (turnover number). The table is reproduced from Guengerich (ed) (1987).

Activity

1,2-Dichloroethane hydroxylation
 N,N-Dimethyl-4-aminoazobenzene N-demethylation
 N,N-Dimethylaniline N-demethylation (and other ring-substituted derivatives)
 Dimethyl benz(a,h)anthracene oxidation
 N,N-Dimethylnitrosamine N-demethylation
 7-Ethoxycoumarin O-deethylase
 2-(N-Ethylcarbamoylhydroxymethyl)furan (covalent binding to protein)
 Ethylmorphine N-demethylation
 Fluroxene (\rightarrow trifluoroethanol) (and other vinyl ethers)
 n-Hexane
 1-Hydroxylation
 2-Hydroxylation
 3-Hydroxylation
 Hexobarbital 3-hydroxylation
 Iodobenzene I-oxygenation (iodosobenzene-dependent)
 4-Isopomeanol (covalent binding to protein)
 Iproniazid oxidation
 Isopropylhydrazine oxidation
 Isosafrole heme adduct formation
 Lasiocarpine oxidation
 Lauric acid (11- and 12-hydroxylation)
 N-Methyl-4-aminoazobenzene N-demethylation
 8-Methylbenz(a)anthracene (8,9 and other epoxidations)
 1-Naphthylamine N-oxidation
 2-Naphthylamine N-oxidation
 p-Nitroanisole O-demethylation
 1-Nitropropane denitrification
 p-Nitrophenetole O-deethylation

Parathion oxidation to
 Paraoxon
 Diethylphosphorothionate
 Diethylphosphate
 trans-1-Phenyl-1-butene oxidation
 Epoxidation
 4-Phenyl-1-butanone
 2-Phenyl-1-butanone
 Progesterone
 16 α Hydroxylation
 Scoparone O-demethylation
 Testosterone
 16 α Hydroxylation
 16 β Hydroxylation
 17 α Hydroxylation
 1,1,2,2-Tetrachloroethane oxidation
 Toluene
 Methyl hydroxylation
 o-Hydroxylation
 m/p-Hydroxylation
 p-Tolylethyl sulfide S-oxygenation
 Trichloroethylene oxidation to chloral
 Epoxidation
 Vinyl chloride
 (products covalently bound to protein)
 Vinylidene chloride oxidation
 Dichloroacetaldehyde
 Chloroacetic acid
 R'-Warfarin 4'-hydroxylation
 Zoxazolamine 6-hydroxylation

cDNA had to be compatible with the unique EcoRI restriction site in the expression vector. A strategy had to be devised that would result in the P450IIB1 cDNA having flanking EcoRI restriction sites. The restriction map of pSP-450 (Figure 3.01) shows an EcoRI site in the multiple cloning site of the SP64 vector while the cDNA has a unique NcoI site spanning the translational initiation codon. The cloning strategy adopted is shown diagrammatically in Figure 3.02. An NcoI restriction digest linearised the spP-450 plasmid while the Klenow fragment of E.coli DNA polymerase I was used to create blunt-ends. Radiolabelled ^{32}P -dATP allowed the Klenow reaction to be followed (Figure 3.03). ^{32}P -dATP labelled EcoRI linkers, d(GGAATTCC), were then blunt-end ligated to the plasmid using T4 DNA ligase. Autoradiography again allowed this reaction to be followed (Figure 3.04). An EcoRI restriction digest produced a cDNA with EcoRI restriction sites flanking the coding sequence. This cDNA was separated from the contaminating sp64 vector using preparative agarose gel electrophoresis, excised from the gel and isolated by centrifugation through a spin-X column. The cDNA was then ligated to EcoRI digested pUC-9 and transformed into E.coli strain JM101. Potential pUC-9/P450IIB1 bearing colonies were selected using the blue/white test with X-gal. TELT DNA mini preparations were carried out on the white colonies and,

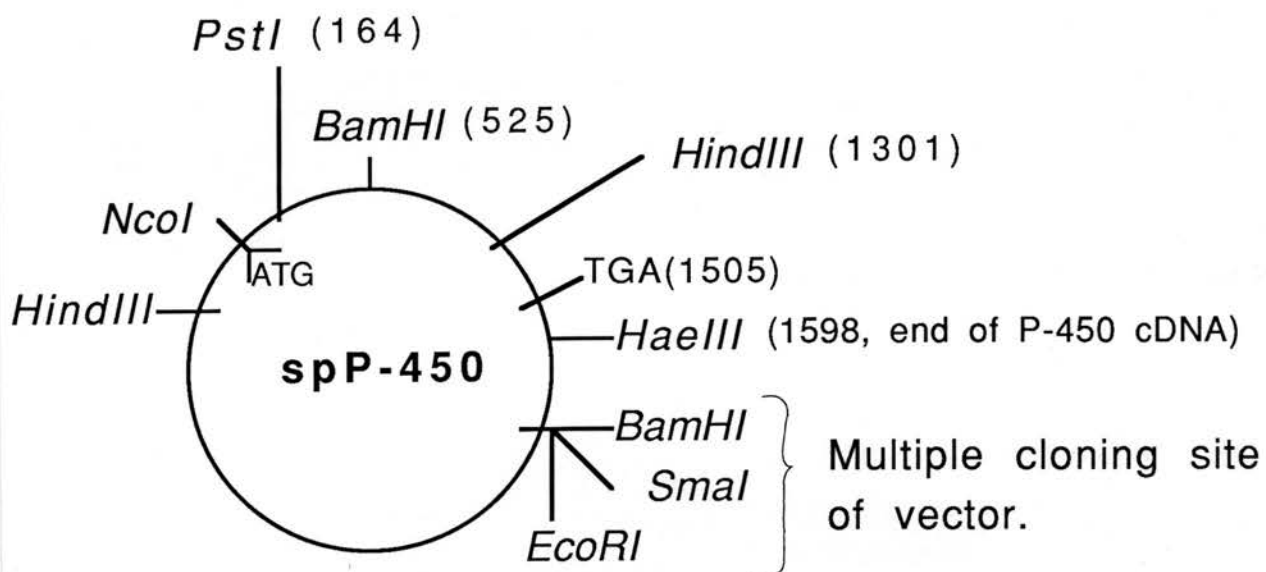


Fig.3.01 Restriction Map of spP-450.

The full-length P450IIB1 cDNA can be released from the sp64 vector using an NcoI/EcoRI restriction digest. The NcoI restriction site overlaps the translational initiation codon and the EcoRI restriction site is found in the multiple cloning site.

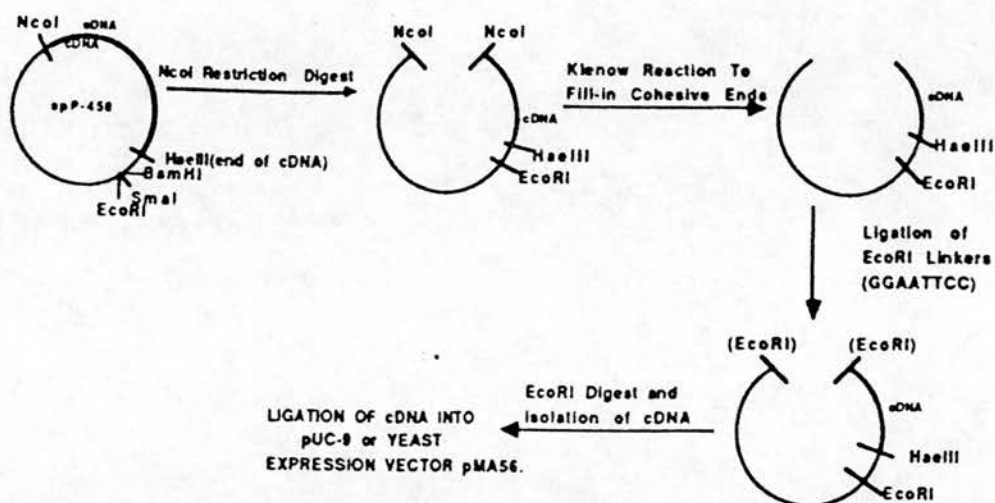
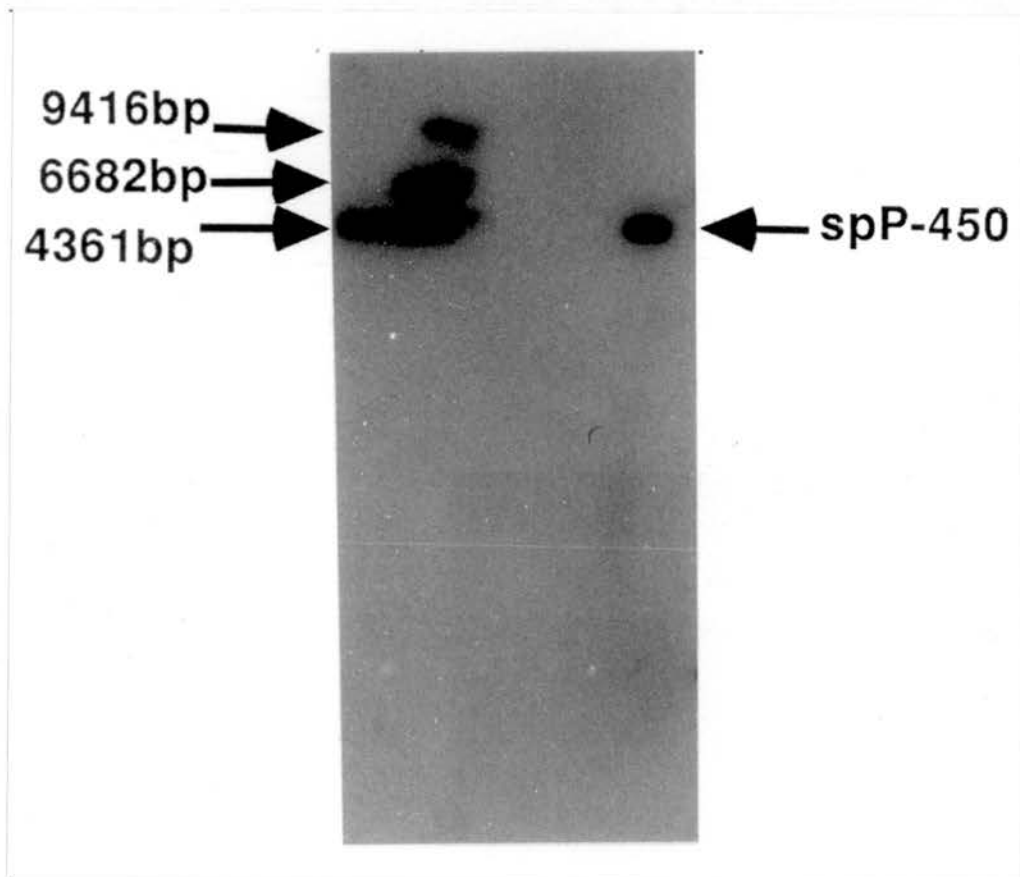


Fig.3.02 Strategy for the cloning of P450IIB1 cDNA into the Yeast Expression Vector pMA56.

The NcoI restriction site overlapping the translational initiation codon of the cDNA was used along with Klenow enzyme and EcoRI synthetic linkers to produce EcoRI restriction sites flanking the P450IIB1 cDNA. The cloning of the P450IIB1 cDNA to produce spP-450 has been detailed elsewhere (Monier et al., 1988). Reactions were as detailed in materials and methods.



**Fig.3.03.Klenow Reaction to Remove the Cohesive Ends from
spP-450.**

The Klenow reaction (section 2.10.3) using ^{32}P -dATP was used to radiolabel the spP-450 plasmid. Preparative agarose gel electrophoresis and autoradiography was used to follow the success of the reaction.

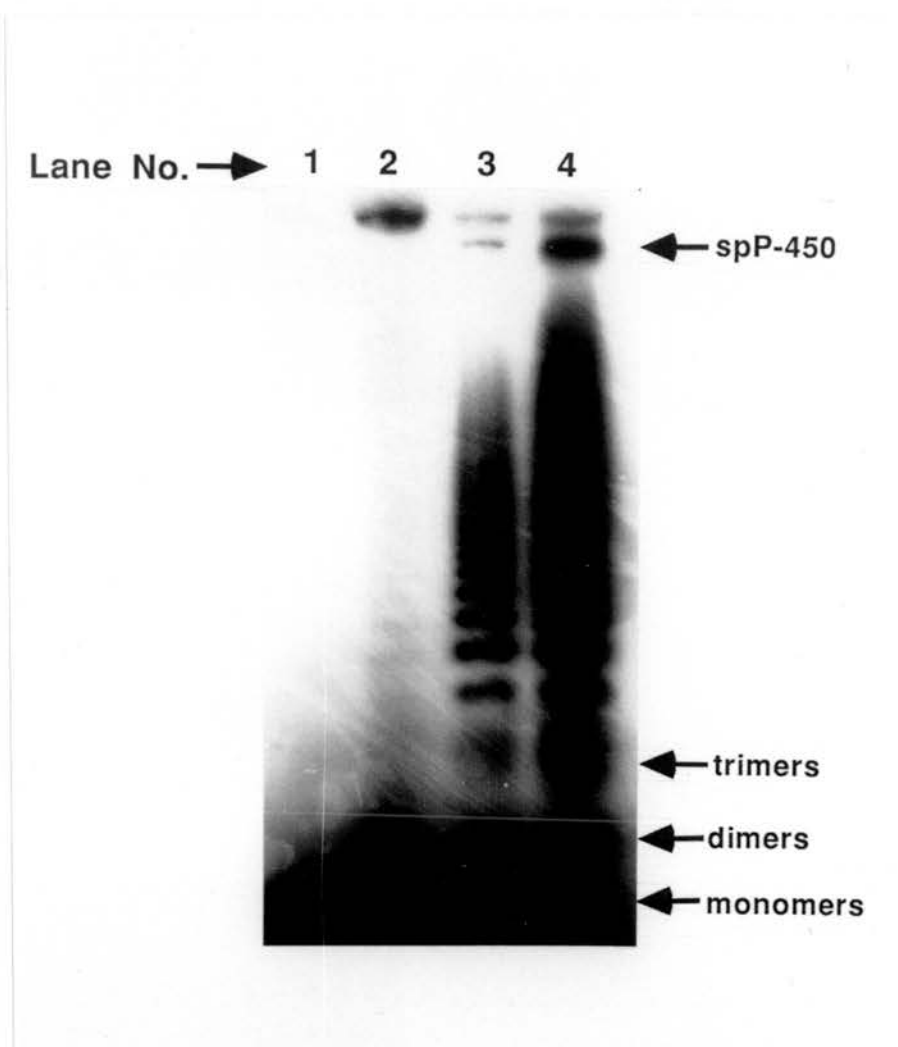


Fig.3.04. PAGE to follow EcoRI Linker Ligations.

An 8% polyacrylamide gel was used to separate the ^{32}P -labelled EcoRI linkers and determine the efficiency of ligation. Unligated linkers were loaded in lane 1; linkers self-ligated in lane 2 and linkers ligated to spP-450 in lanes 3 and 4. A ladder effect from monomers upwards is a sign of success in the reaction. This is only observed where T4 DNA ligase has been added to the reaction (lanes 2-4).

after EcoRI digestion, the plasmids were subjected to preparative agarose gel electrophoresis. The results are shown in Figure 3.05. As can be seen, only the plasmid in lane 2 appears to have a band that comigrates with the P450IIB1 cDNA released by an EcoRI/NcoI double digest of pSP64 (lane 14). To determine if this band is in fact P450IIB1 a Southern transfer was performed and the nitro-cellulose filter probed with ^{32}P labelled P-450IIB1. The result is shown in Figure 3.06. Only two tracks give a positive signal for P450IIB1 the control in lane 14 and the EcoRI digested PUC-9/P450IIB1 plasmid DNA in lane 2. This unequivocally demonstrated that the pUC-9 plasmid in lane 2 has the P450IIB1 insert. This plasmid was designated pUC/PB3a and used in all further studies to sub-clone the P450IIB1 cDNA into the yeast expression vector.

3.3 Removal of Terminal Phosphates of pMA56

The pMA56 yeast expression vector (from J.D. Beggs, Dept. Molecular Biology, University of Edinburgh) is shown in Figure 2.01. This plasmid is a 2 μ based shuttle vector that can be propagated in both E.coli and S.cerevisiae.

The possession of a pBR322 origin of replication and a β -lactamase gene allows replication and selection in E.coli. For replication in yeast pMA56 has the 2 μ origin

Fig.3.05. Restriction Digestion of Possible pUC/PB_{3a} Clones.

EcoRI restricted pUC/PB_{3a} DNA (1μg) isolated from various E.coli JM101 transformants was loaded in lanes 2-13. Hind III digested Phage lambda DNA (1μg) was loaded in lane 1 and EcoRI/NcoI digested spP-450 DNA loaded in lane 14 as a size marker for the P450IIB1 cDNA. After preparative agarose gel electrophoresis only the DNA loaded in lane 2 gave a band comigrating with the P450IIB1 (PB_{3a}) cDNA released from the spP-450 vector (lane 14) .

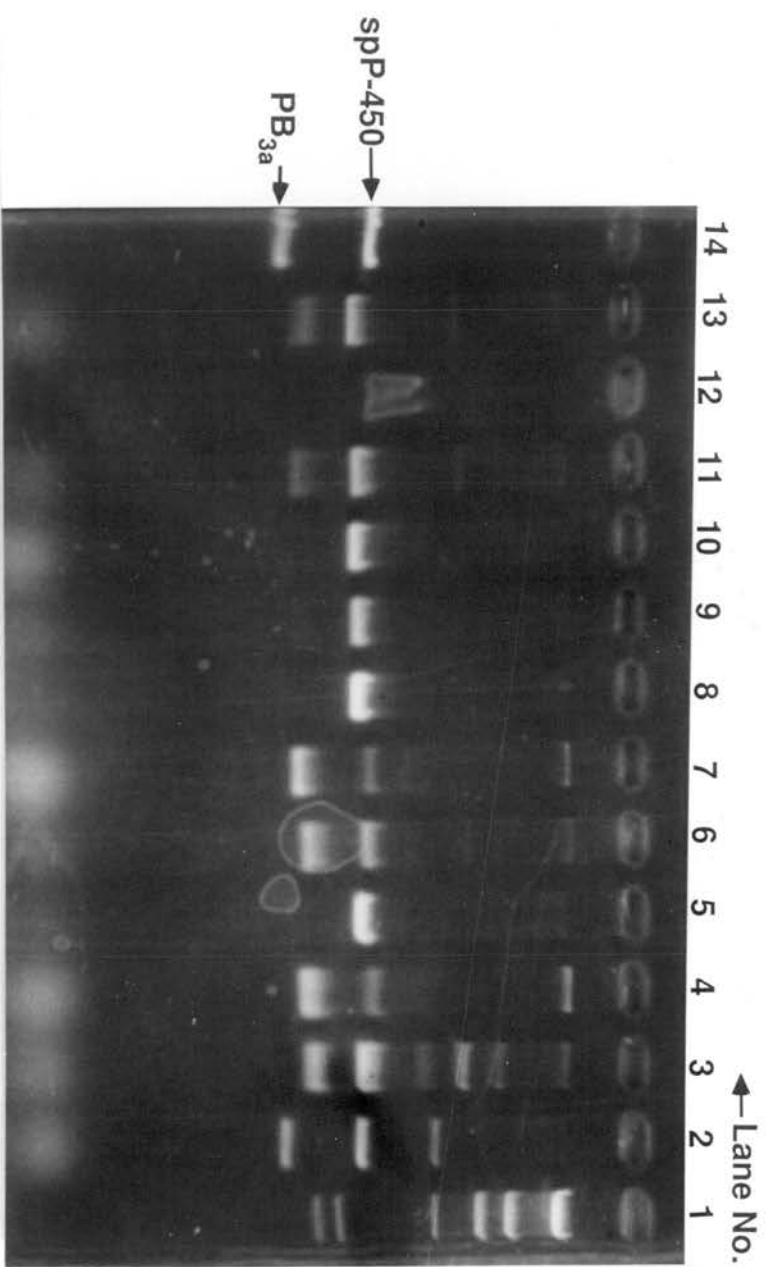


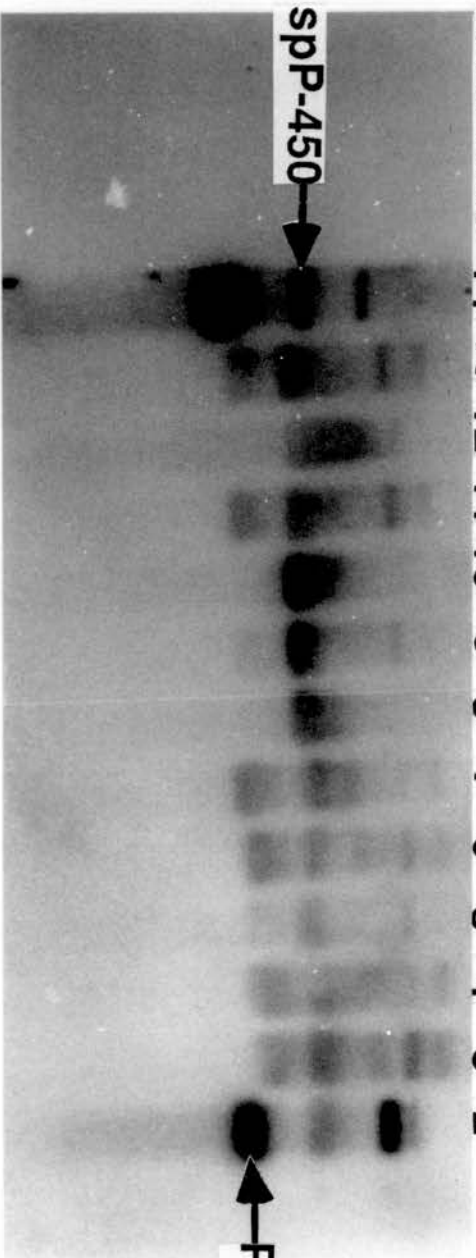
Fig.3.06. Southern Analysis of EcoRI Digested pUC/PB_{3a} Clones.

³²P-labelled P450IIB1 cDNA isolated from the spP-450 vector was used to probe the agarose gel in fig.3.05 after the DNA had been transferred to nitrocellulose. Again only the DNA in lane 2 along with the control in lane 14 gave a cross-reacting band with the P450IIB1 probe. The plasmid in lane 2 was designated pUC/PB_{3a}.

Lane No. 14 13 12 11 10 9 8 7 6 5 4 3 2

spP-450

PB_{3a}



of replication (Beggs et al, 1976) and TRP1 gene to allow the complementation of the trp1 host mutation giving a positive selection for the plasmid. The vector has the strong constitutive alcohol dehydrogenase promoter, ADC1 (Hitzeman, 1981; Ammerer, 1983).

In order to decrease the number of self-ligated vectors bacterial alkaline phosphatase was used on EcoRI digested DNA to remove the terminal phosphate groups of the vector. This prevents the vector from religating in the absence of a cDNA insert. To test the success of the phosphatase reaction the vector was ligated to itself or to EcoRI digested pUC-18. The products were then subjected to agarose gel electrophoresis. This is shown in Figure 3.07. The plasmid produced from this successful reaction was designated p56-RIBAP and used for subsequent ligation to the P450IIB1 cDNA subcloned into pUC-9.

3.4 Subcloning of P450IIB1 into pMA56

The P450IIB1 cDNA was isolated from pUC-9 via an EcoRI restriction digest, agarose gel electrophoresis and centrifugation through a spin-X column. The cDNA was then ligated to p56-RIBAP and transformed into the E.coli strain DH1. The colonies produced were subjected to the colony screening assay of Grunstein & Hogness (1975) with ³²P-labelled P450IIB1 cDNA as a probe. The result of

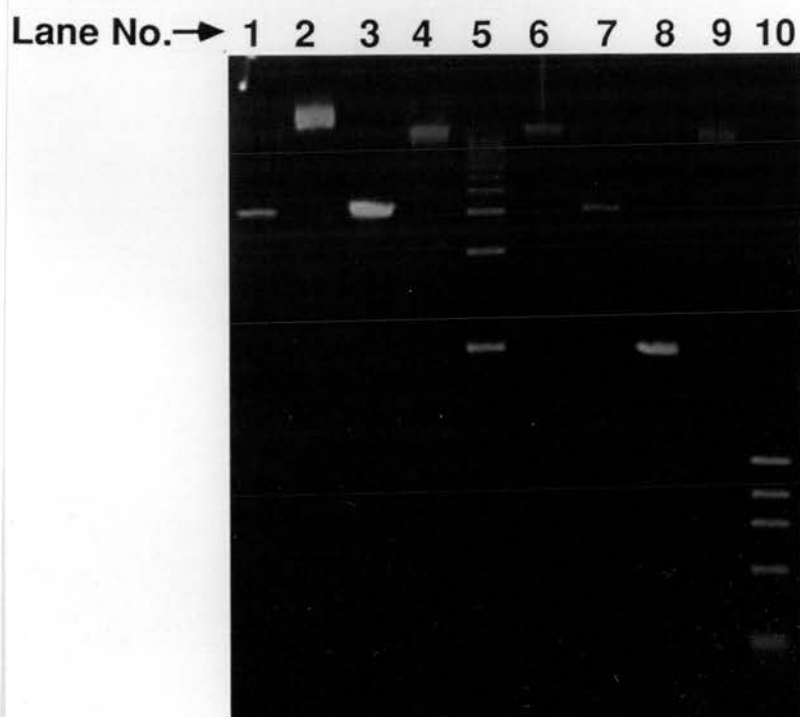


Fig.3.07. Bacterial Alkaline Phosphatase (BAP) Treatment of EcoRI Digested pMA56.

The 5'- phosphate groups of EcoRI digested pMA56 were removed using BAP to prevent self-ligation and increase subcloning efficiency (section 2.9.2). EcoRI digested pUC-18 was used in conjunction with preparative agarose gel electrophoresis to test the plasmid produced (p56-RI BAP). Lanes 1-3 contain p56-RI BAP, undigested pMA56 and EcoRI digested pMA56 (p56-RI) respectively. Lanes 4 and 6 contain p56-RI BAP ligated to pUC-18 and lane 5 contains p56-RI ligated to pUC-18. Lanes 7 and 9 contain self-ligated p56-RI BAP and EcoRI digested pUC-18 respectively. Lane 8 contains unligated EcoRI digested pUC-18. The ϕ X174 Hae III DNA size Marker was loaded in lane 10. The p56-RI BAP vector gives a much cleaner ligation to pUC-18 when compared to p56-RI (lanes 4 and 6 vs. lane 5). This result shows that the BAP treatment has reduced the efficiency of self-ligation without hampering the ligation of insert DNA.

which is seen in Figure 3.08. This gave six possible recombinant plasmids. DNA was prepared from each and subjected to EcoRI digestion to determine if the P450IIB1 cDNA was present. At the same time a BamHI digest to determine the orientation of the cDNA within the vector was carried out. These digests are shown in Figures 3.09.1 and 3.09.2. The plasmid shown in lane 2 in Figure 3.09.1 and lane 3 in Figure 3.09.2 was used for all further expression studies since it possessed both the P450IIB1 cDNA and the correct, 2.0Kb, fragment from the BamHI digestion diagnostic of the orientation required for protein expression. This plasmid was designated p56/3a. The parental, pMA56 vector, was designated p56 par.

3.5 Cytochrome P-450 Estimation in Various *S.cerevisiae* Strains

The object of the study undertaken was to express a mammalian P-450 in yeast and to examine the potential of the protein to activate promutagens. A strain that had a low endogenous P-450 level was required since it was desirable to have as low a background activation rate as possible, i.e. metabolism catalysed by endogenous yeast P-450 needed to be minimal. The strain also had to be trp1 to allow for the selection of the p56/3a vector. The strain had also to produce reductase to allow the

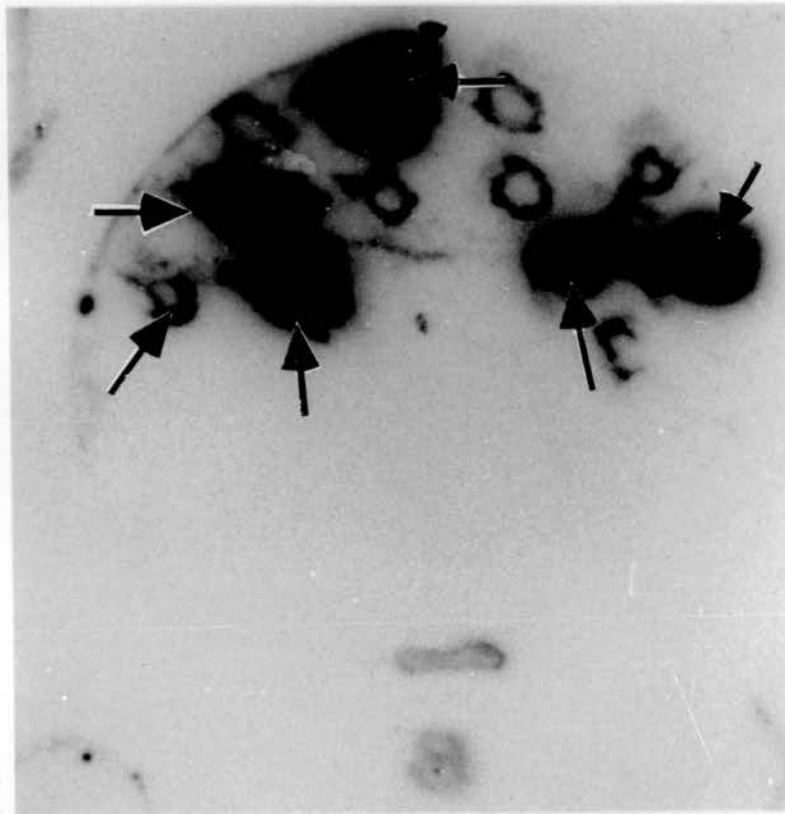


Fig.3.08. Colony Hybridisation Analysis on Transformed E. coli DH1 Cells.

The colony hybridisation method of Grunstein & Hogness (1975) (section 2.15) was used to determine which of the transformed E. coli DH1 colonies contained the p56/3a vector. Using ^{32}P -labelled P450IIB1 cDNA as a probe followed by autoradiography this gave six possible p56/3a carrying colonies (marked by arrow).

Fig.3.09. Restriction Enzyme Analysis on Possible p56/3a Constructs.

Plasmid DNA was isolated from the six possible p56/3a carrying DH1 colonies identified in fig.3.08 as described in section 2.4.2. This DNA was subjected to restriction analysis and agarose gel electrophoresis to determine if the P450IIB1 cDNA (PB_{3a}) was present (fig.3.09.1) and the orientation of the insert (fig.3.09.2). The EcoRI restriction digest identified four plasmids carrying the P450IIB1 cDNA (lanes 2,3,4 and 6 respectively in fig.3.09.1). The BamHI digest identified the diagnostic 2.0Kb fragment required for expression in lanes 3,4 and 8 in fig.3.09.2. In both fig.3.09.1 and 3.09.2 Hind III restricted phage lambda DNA was used as a DNA size marker (lane 1).

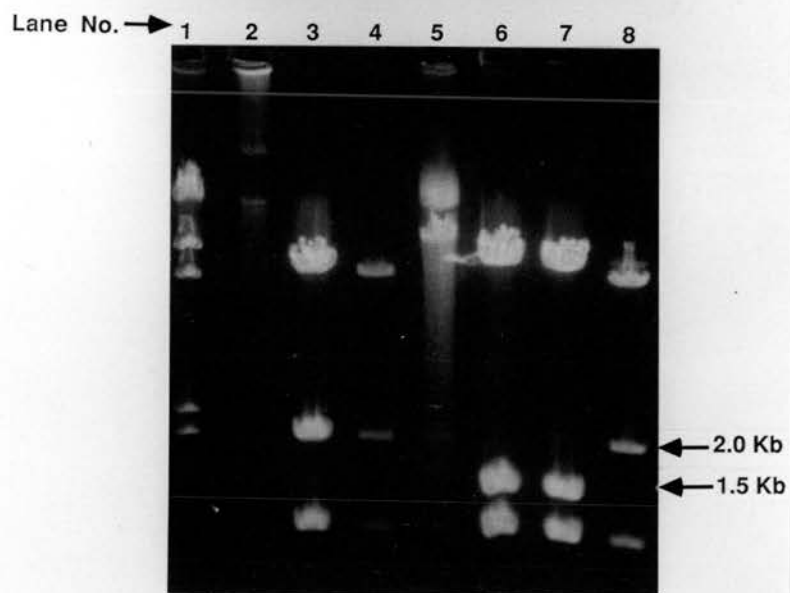
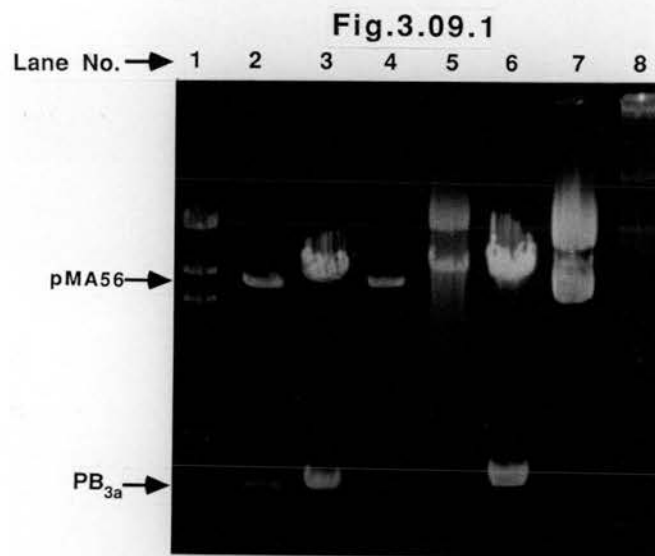


Fig.3.09.2

mammalian P-450 to function. The P-450 levels of various trp1 strains were determined from their CO binding spectra. Strains were cultured to late logarithmic phase. The strains assayed were 1/ RH971; 2/ 1-103-50; 3/ 1.16B; 4/ ENYWA-1A; 5/ UTL-7A; 6/ YS18; 7/ KY118. Strains 1-6 were a generous gift from A. Hinnen of the Ciba-Geigy Biocentre, Basle, Switzerland and KY118 a gift from J.D. Beggs. The CO-binding spectra of each strain are shown in Figures 3.10A through 3.10G. The strains 1-103-50; 1.16B; ENYWA-1A; UTL-7A and YS18 all show significant Soret peaks at 450nm whereas strains RH971 and KY118 show no such peak. The levels of P-450 were then calculated using an extinction coefficient (450-490) of $91\text{mM}^{-1}\text{cm}^{-1}$ and the results shown in Table 3.2. The strains RH971 and KY118 have very low levels of P-450 (7.32 pmol/OD). This capacity to produce P-450 (although at low levels) also suggests the ability to produce yeast reductase which would give an active mammalian P-450. From this it was decided to use the KY118 strain as the recipient for the p56/3a vector.

3.6 P450IIB1 mRNA Expression

The lithium acetate method of Itoh et al (1983) was used to transform the S.cerevisiae strain KY118 either with p56/3a or p56 par. The resultant colonies were then inoculated into supplemented synthetic SD medium and

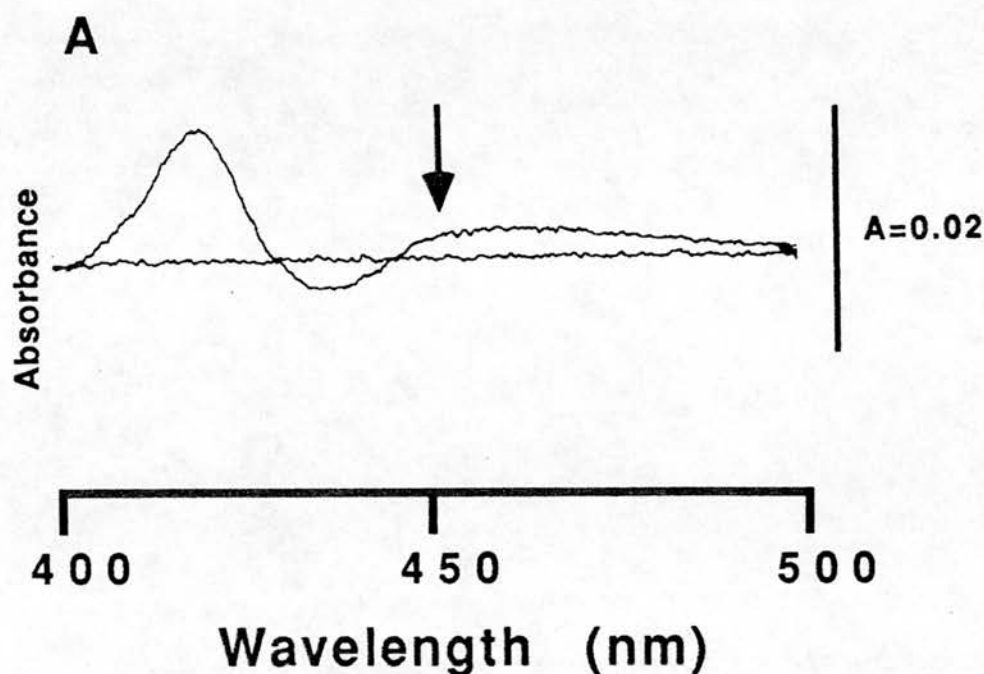
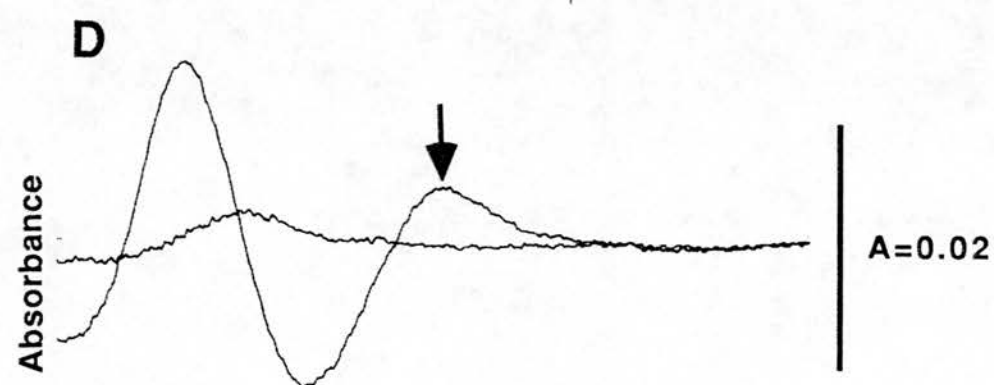
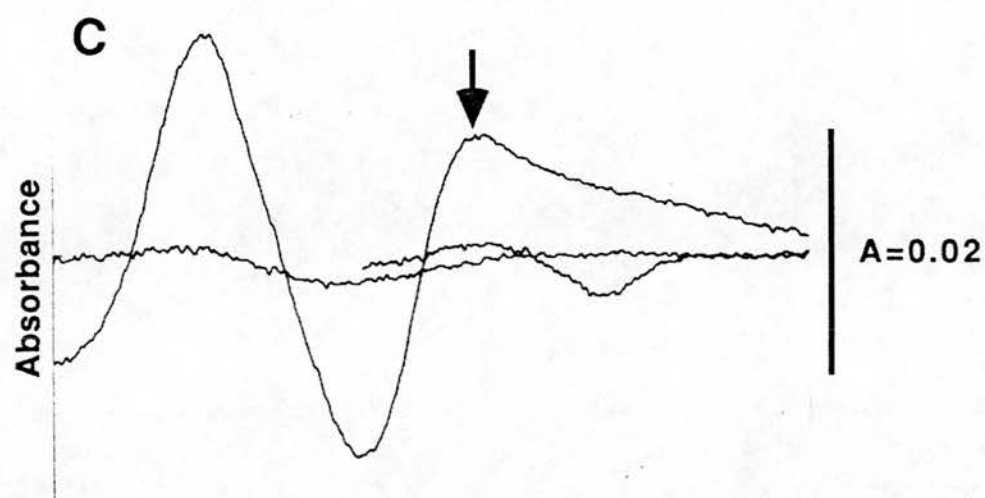
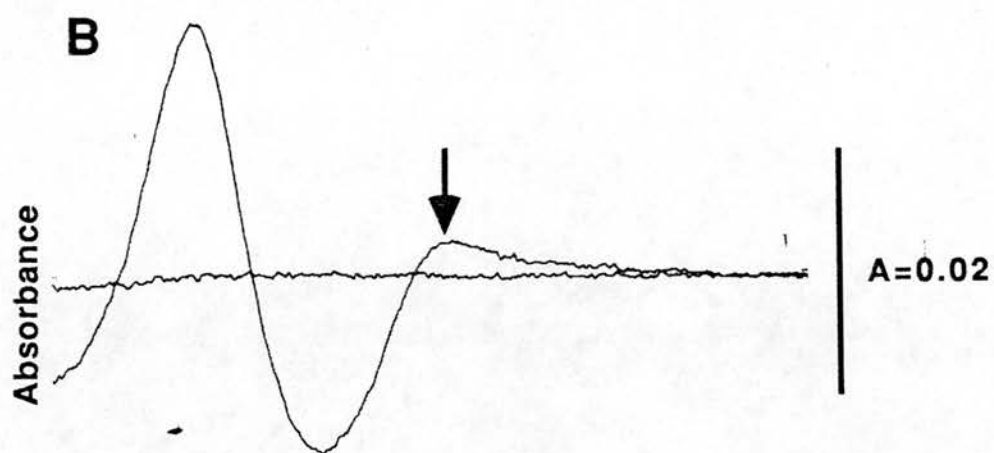


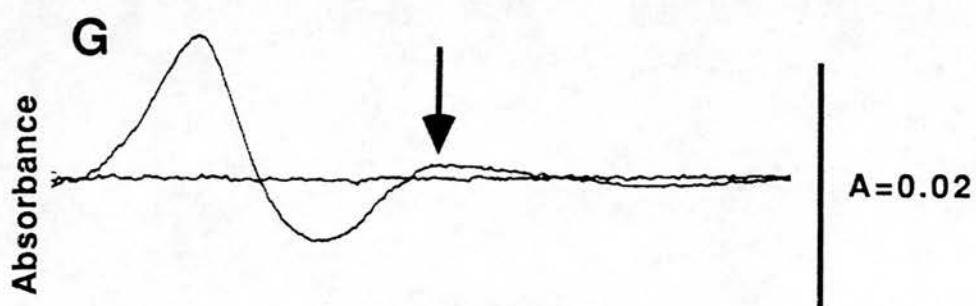
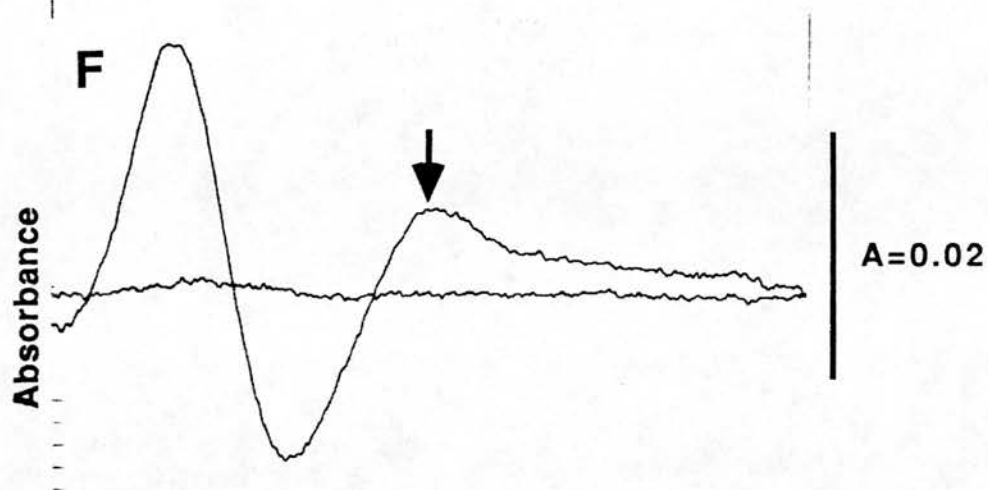
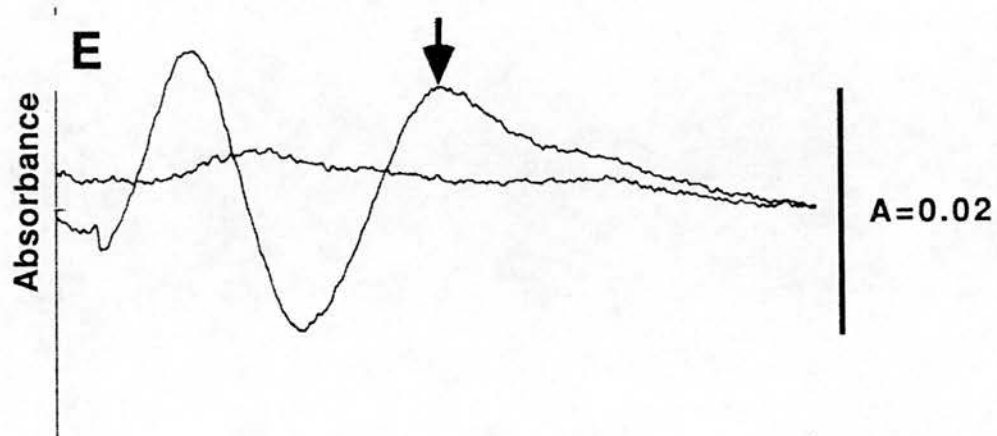
Fig.3.10 Cytochrome P-450 Levels in Various *S.cerevisiae* Strains.

Carbon monoxide binding spectra were carried out on whole yeast cells according to the method of Omura & Sato (1964) (section 2.17.2). Various *S.cerevisiae* strains were examined all of which had the trp1 mutation. The strains used were RH971 (A); 1-103-50 (B); 1.16B (C); ENYWA-1A (D); UTL-7A (E); YS18 (F); KY118 (G). The solet maximum at 450nm is marked with an arrow in each case. An extinction coefficient (450-490nm) of $91\text{mM}^{-1}\text{ cm}^{-1}$ was used to determine the level of P-450 in each strain (table 3.2).



400 450 500

Wavelength (nm)



400 450 500

Wavelength (nm)

Strain	Level of P-450 (pmol/OD)
RH971	9.2
1-103-50	48.7
1.16B	100.4
ENYWA-1A	74.8
UTL-7A	122.7
YS18	80.9
KY118	9.2

Table 3.2 Cytochrome P-450 Levels in various S.cerevisiae Strains.

The P-450 spectra in Fig.3.10 were used to determine the level of P-450 in each of the trp1 mutant strains. An extinction coefficient (450-490nm) of $91\text{mM}^{-1}\text{cm}^{-1}$ was used. P-450 levels were calculated as $(\text{Aborbance at } 450\text{nm}/0.091) \times 1000/\text{OD}_{600}$.

harvested in late logarithmic phase. RNA was extracted from both strains and subjected to electrophoresis through a formaldehyde denaturing gel. RNA was then transferred to nitro-cellulose and probed with ^{32}P -labelled P-450IIB1 cDNA. This Northern blot is shown in Figure 3.11. The KY118 strain carrying p56/3a shows a cross-reacting band to the ^{32}P -labelled P450IIB1 cDNA but no such band is observed in the track which the 56 par strain was loaded. The smearing effect seen in the P450IIB1 RNA band produced from the p56/3a vector is probably due to the absence of a transcription termination sequence in pMA56. This was removed from the vector pAAR6, along with an ARS1 sequence, to produce pMA56 (Parent *et al.*, 1985).

3.7 P450IIB1 Protein Expression

3.7.1 Level of P450IIB1 Protein Expression

The p56/3a and p56 par carrying strains were again inoculated into supplemented synthetic SD medium and allowed to grow into stationary phase before being harvested. Cells were homogenised and 50ug of protein from each was subjected to Western blotting. Polyclonal antibodies raised against rat P450IIB1 or P450IIC proteins were used as probes along with ^{125}I -labelled protein A. The anti P450IIC antisera was used as a negative control since it would not be expected to

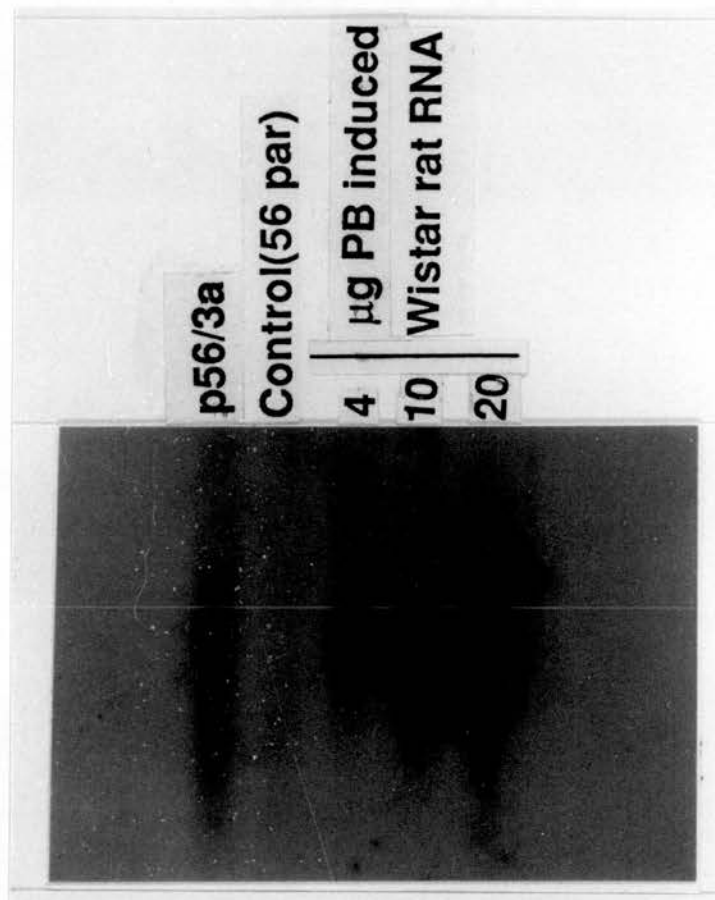


Fig.3.11. Expression of P450IIB1 mRNA in S. cerevisiae

Strain KY118 transformed With p56/3a or p56par.

Total RNA was isolated from KY118 containing either the p56/3a (lane 1) or p56par(lane 2) vectors. In lanes 3-5: 4, 10, and 20µg of PB-induced Wistar rat RNA was loaded. Samples were analysed as in section 2.13.

react with the P450IIB1 protein as they are only 50% similar at the protein level.

Only the p56/3a transformed strain contained a protein band that reacted with the anti P450IIB1 antibody (Figure 3.12). The protein observed had the same mobility as the protein standard isolated from rat liver. No cross-reacting bands were observed in the p56 par transformed strain or the p56/3a strain probed with the antibody to P450IIC (Figure 3.12). Thus the P450IIB1 antibody was specific for the protein produced from the p56/3a vector and does not cross-react with endogenous yeast proteins. Densitometric scanning of the band intensity and comparison with P450IIB1 protein standards indicated that between 0.1 - 0.2% of total yeast protein was P450IIB1.

3.7.2 Subcellular Localisation of P450IIB1 Protein

Subcellular fractions were prepared from the yeast strains carrying the p56/3a and p56 par vectors using the French press method detailed in Section 2.15.1. These fraction were subjected to SDS-page then Western blotting using P450IIB1 antisera and ^{125}I -labelled protein A. This is shown in Figure 3.13. In the rat liver the P450IIB1 protein is localised in the endoplasmic reticulum (equivalent to the microsomes track in Figure 3.13) by the insertion-halt-transfer sequence

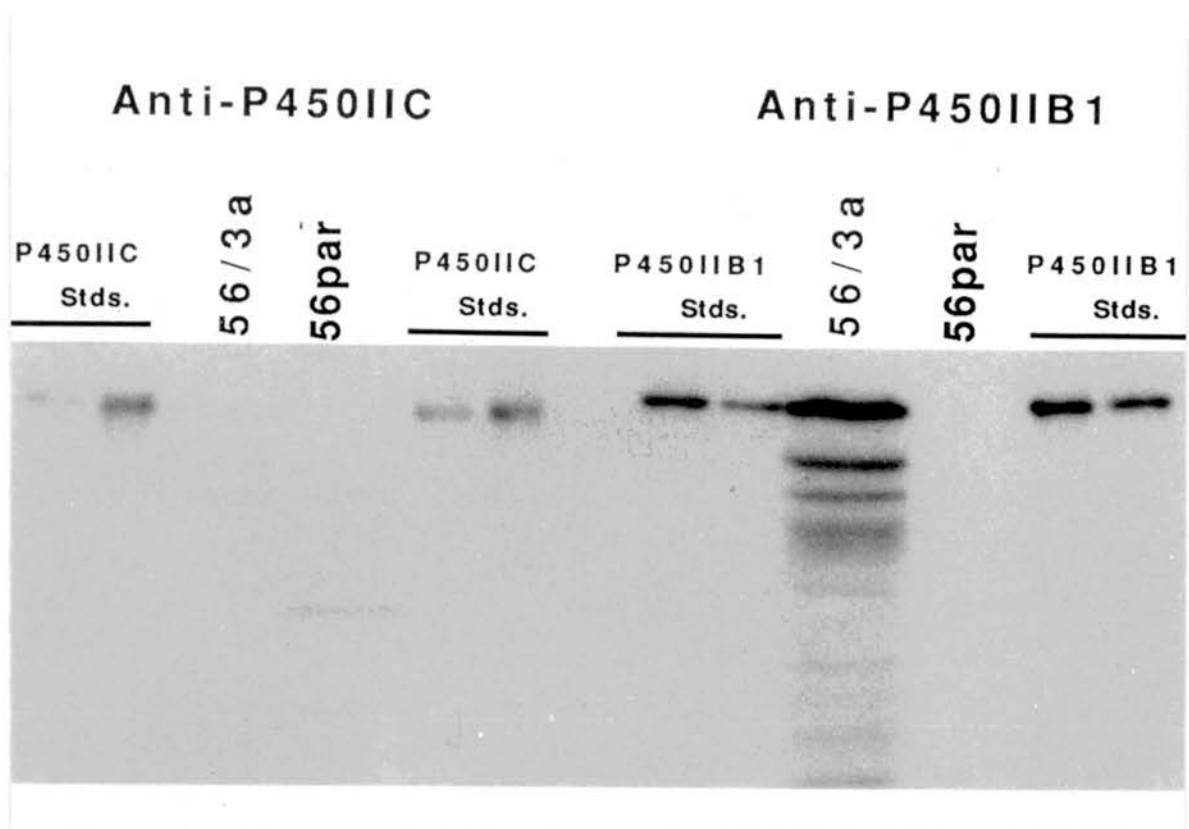


Fig.3.12. Expression of P450IIB1 Protein in S.cerevisiae.

SDS-page followed by Western blot analysis (section 2.19) was carried out on whole cell extracts of p56/3a or p56par transformed KY118. Antibodies raised against the rat P450IIB1 or P450IIC proteins were used to identify the P450IIB1 protein and test the specificity of the recognition respectively. Only the p56/3a transformed strain produces a protein that cross-reacts with the P450IIB1 antibody (the lower molecular weight bands are due to the degradation of the P450IIB1 protein). When the P450IIC antibody is used no such band is observed thus the P450IIB1 antibody is specific for the P450IIB1 protein. The p56par transformed strain shows no cross-reacting band with either antibody. 50µg of protein was loaded per track.

encoded by the amino terminal 20 amino acid residues of the protein (Monier et al, 1988). The question thus posed was whether this signal sequence would function similarly in S.cerevisiae to localise the protein to the endoplasmic reticulum or would the P-450 protein be found free in the cytosol. From the Western blot (Figure 3.13) it is obvious that there is only a faint cross-reacting band in the track containing cytosol showing that the P450IIB1 protein is not free within the cytoplasm. Whereas there is a highly reactive band found in the track loaded with the microsomal fraction. Thus the P450IIB1 protein produced from p56/3a is localised to the yeast endoplasmic reticulum implying that the insertion-halt-transfer sequence is functional within S.cerevisiae. A large quantity of P450IIB1 protein, around 1/5 - 1/10 of that in the microsomal fraction was found in the cell debris. This may demonstrate a limitation in the use of the French press to disrupt yeast cells. This may be due to the endoplasmic reticulum being attached to the cell membrane which is sedimented during the first clearing spin.

3.8 Determination of P-450 Reductase Levels in Strain Carrying p56/3a

As previously discussed (Section 1.1) cytochrome P-450's receive electrons from P-450 reductase. The yeast

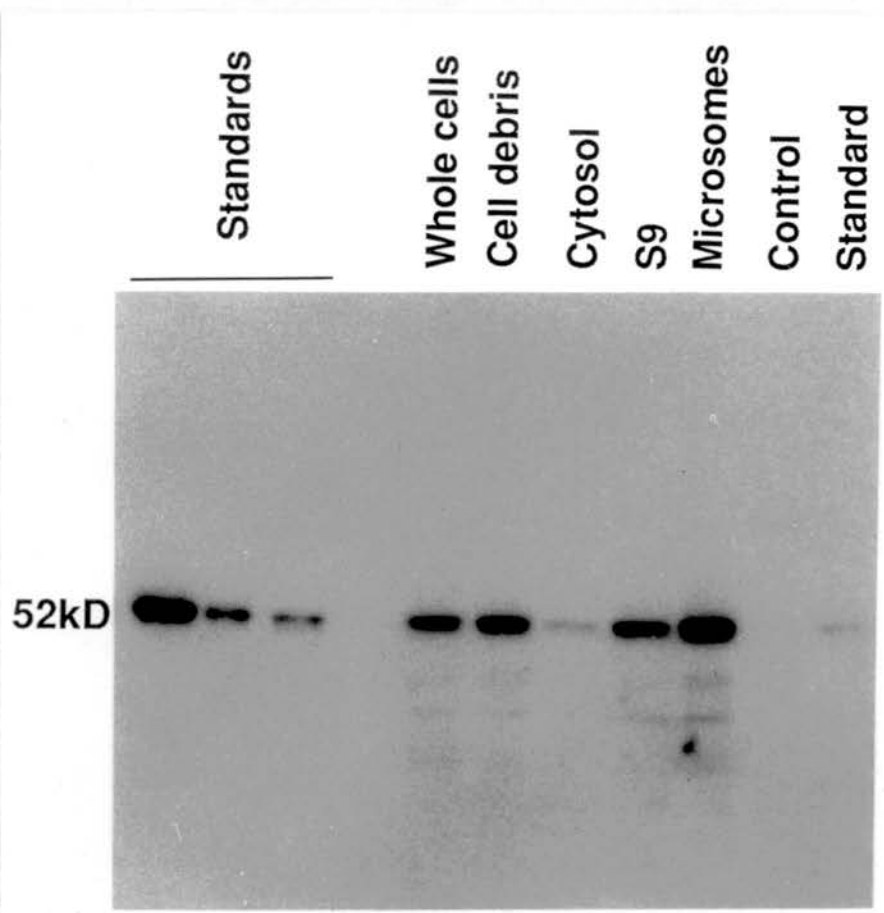


Fig.3.13. Subcellular Localisation of P450IIB1 Protein in S. cerevisiae.

SDS-page followed by Western blot analysis of control yeast cultures and yeast transformed with the vector containing P450IIB1 cDNA (p56/3a) was carried out on subcellular fractions using an antibody raised against the purified P450IIB1 protein. It can be seen that the 52kD protein associated with P450IIB1 is predominantly localised in the microsomal fraction. Western blots were carried out as described in section 2.19. with 25µg of protein loaded in each track.

enzyme has been shown to have the capacity to couple to a mammalian P-450 enzyme (Oeda et al, 1985). On this basis it was necessary to establish the level of reductase present in the KY118 strain. The simplest determination of the reductase level in yeast is to use the cytochrome c assay. This reaction is not specific for P-450 reductase but detects all cellular reductases. Various subcellular fractions of the 56/3a strain were assayed for reductase activity and compared to control and PB-induced rat liver microsomes (Table 3.3). The level of reductase is highest within the microsomal fraction suggesting this is P-450 reductase. The level found in the yeast microsomal fraction is approximately equal to that found in control rat liver microsomes and 50% of that in PB-induced microsomes. The level of reductase activity associated with the yeast microsomal fraction should give P450IIB1 activity.

3.9 Optimisation of P450IIB1 Protein Expression

It has been shown previously (Wiseman et al, 1985) that the level of yeast P-450 can be affected by the concentration of glucose present in the growth medium. Levels of < 2.5% glucose gave rise to 'low glucose behaviour' which reduces the level of P-450 obtained. In glucose concentrations of >5% there is late accumulation of P-450 to a higher level ('high glucose' behaviour).

Yeast Subcellular Fraction	Reductase Activity
Whole cell	44.9
Cell Debris	21.4
Mitochondria	30.9
Cytosol	10.4
Microsomes	135.6
Control Rat Liver Microsomes	168.0
PB-Induced Rat Liver Microsomes	260.3

Table 3.3 Reductase Level in S.cerevisiae Subcellular Fractions.

The P-450 reductase assay (section 2.17.3) was used to estimate the level of endogenous yeast reductase in the subcellular fractions of the P450IIB1 expressing strain. Reductase activity is expressed in units /mg of protein, using an extinction coefficient of $18.7 \text{ mM}^{-1}\text{cm}^{-1}$. For comparison the reductase level found when the assay was carried out using control and PB-induced rat liver microsomes is included.

To find the glucose concentration that gave the maximum production of P450IIB1 protein and minimal endogenous P-450 levels the 56/3a and 56 par strains were grown in supplemented synthetic SD medium containing 1% casamino acids and a range of glucose concentrations (1,2,5, 20%). CO spectra were determined from late logarithmic cultures to estimate P-450 content (Figures 3.14 A-D, Figure 3.15 A-D and Table 3.4). The glucose concentration at which the P450IIB1 level was optimal and the endogenous P-450 level minimum was 2%. Higher concentrations of glucose had no effect in inducing endogenous P-450 levels but decreased the level of P450IIB1 by 50%. It is unclear why there should be a variation in P450IIB1 at differing glucose concentrations is unknown but for all further experiments a glucose concentration of 2% was used.

3.10 Functional Assay of P450IIB1 Protein

The requirement of P-450 for reductase made it necessary to determine if the P450IIB1 protein measured by CO binding (Section 3.9) was functional, i.e. could couple to the yeast reductase to produce a functional monooxygenase system.

There are many reactions that can be used as a measure of P450IIB1 activity, for example, the O-deethylation of 7-ethoxycoumarin or benzphetamine N-demethylation (Wolf et al, 1986). However, the method

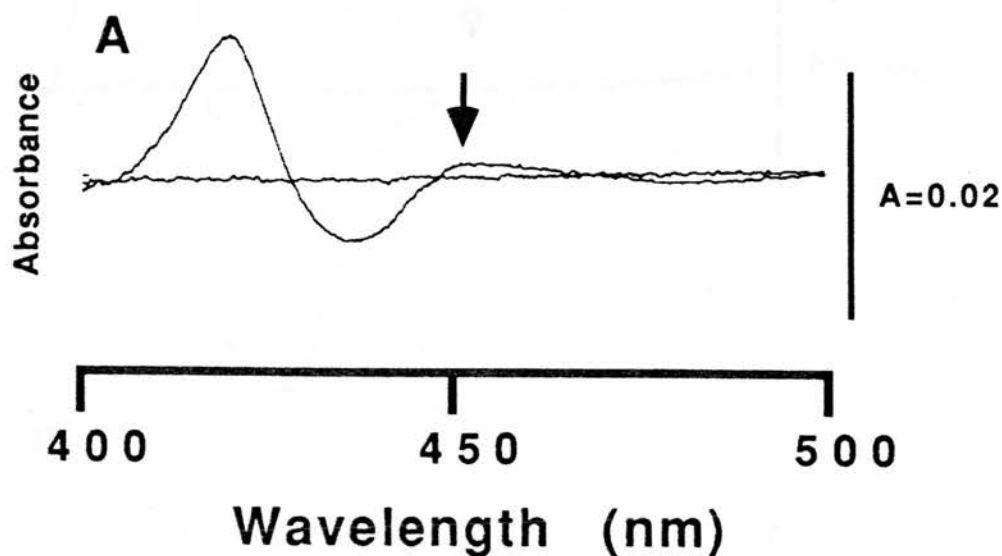


Fig.3.14 Effect of Changes in Glucose Concentration on KY118 Cytochrome P-450 Levels.

The carbon monoxide binding spectra of whole wild type KY118 cells grown in different glucose concentrations was determined according to the method of Omura & Sato (1964) (section 2.17.2). The glucose concentrations used were 1% (A); 2% (B); 5% (C); 20% (D). P-450 levels were calculated using an extinction coefficient (450-490 nm) of $91\text{mM}^{-1}\text{cm}^{-1}$ (table 3.4). The arrows indicate the Soret maximum at 450nm.

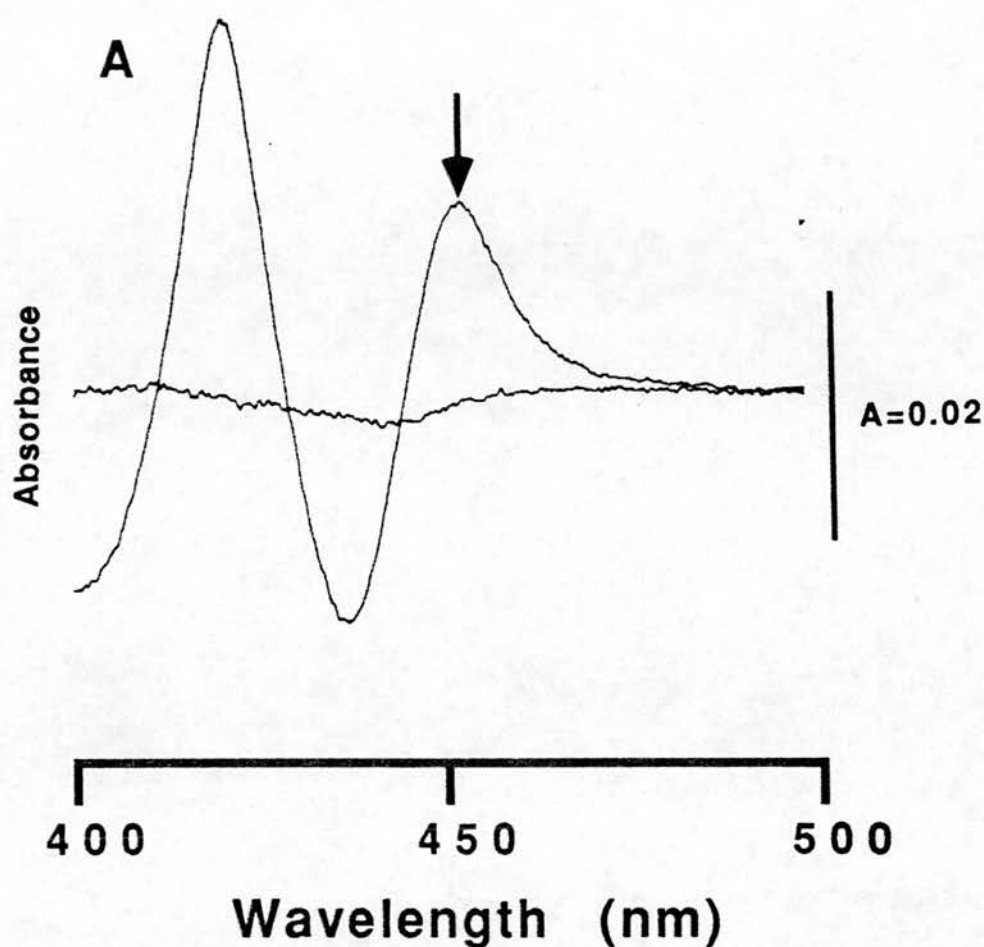
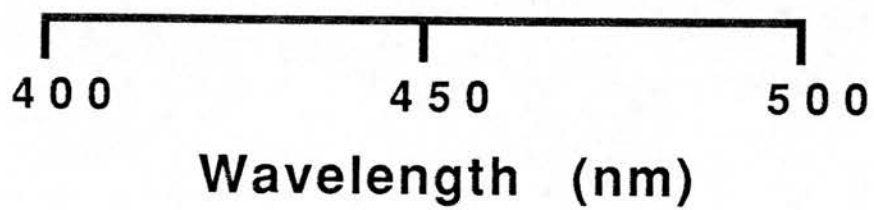
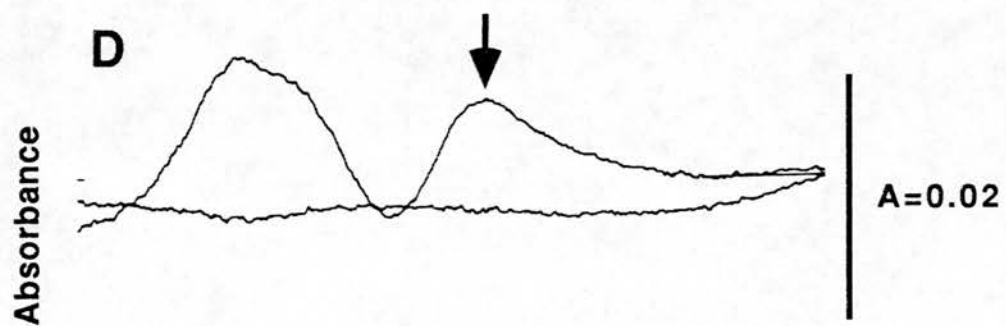
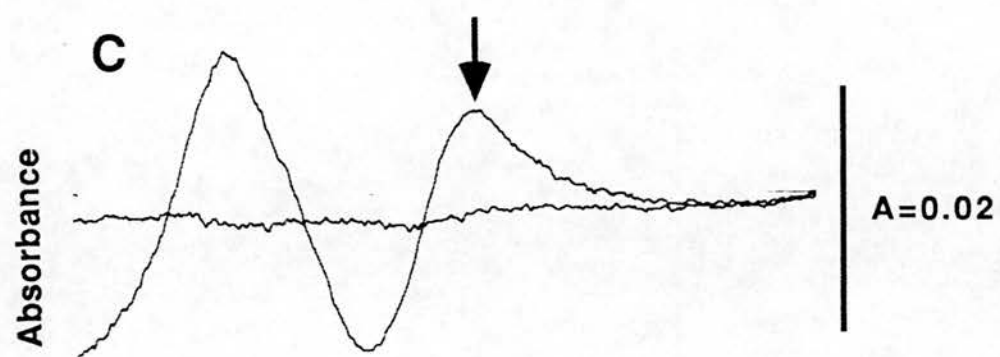
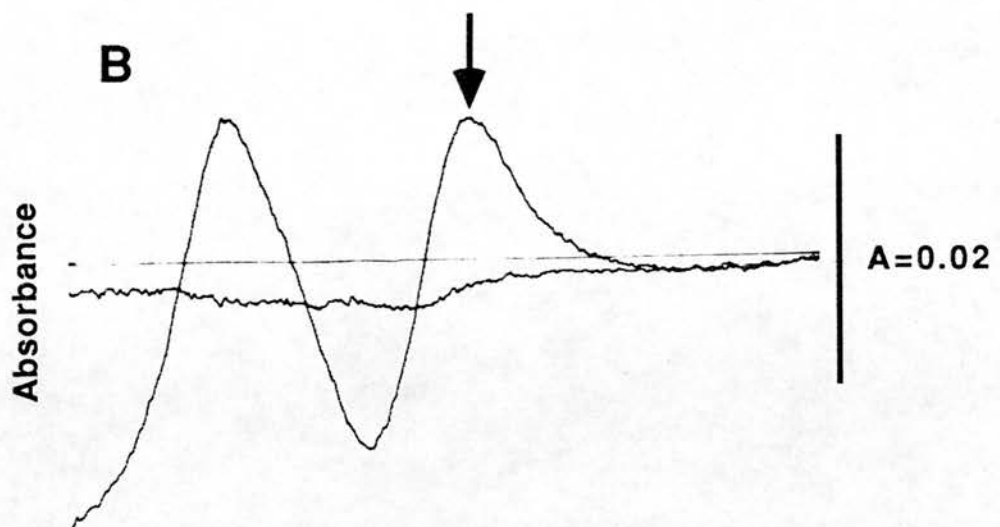


Fig.3.15 Effect of Changes in Glucose Concentration on P450IIB1 Protein Levels.

The carbon monoxide binding spectra of whole P450IIB1 expressing KY118 cells grown in different glucose concentrations was determined according to the method of Omura & Sato (1964) (section 2.17.2). The glucose concentrations used were 1% (A); 2% (B); 5% (C); 20% (D). P-450 levels were calculated using an extinction coefficient (450-490 nm) of $91\text{mM}^{-1}\text{cm}^{-1}$ (table 3.4). The arrows indicate the solet maximum at 450nm.



Glucose concentration (%)	Level of P-450 (pmol/ OD)
1	227.7
2	219.8
5	126.3
20	120.7

**Table 3.4 Cytochrome P-450 Levels in the P450IIB1 Expressing Strain
When Grown in Different Glucose Concentrations.**

The P-450 spectra in Fig.3.14 were used to determine the level of P-450 when the strain expressing the P450IIB1 protein (56/3a) was grown in medium with glucose concentrations of 1,2,5 or 20%. An extinction coefficient (450-490nm) of $91\text{mM}^{-1}\text{cm}^{-1}$ was used. P-450 levels were calculated as $(\text{Absorbance at } 450\text{nm}/0.091) \times 1000/\text{OD}_{600}$.

used was the O-dealkylation of benzyloxyresorufin to resorufin (Burke & Mayer, 1974). Resorufin is a fluorescent product that can be measured spectrophotometrically such that the rate of resorufin appearance is a direct determinant of P450IIB1 activity.

Yeast microsomal fractions were prepared from both the 56/3a and 56 par strains. The O-dealkylation of benzyloxyresorufin was then determined using approximately 50µg of microsomal protein. The assays were carried out a minimum of five times and a representative trace obtained from both strains is shown in Figure 3.16. Metabolism was only detected in the 56/3a strain with the level of O-dealkylation of substrate being 0.16 nmol/min/mg microsomal protein.

3.11 Mutation Assays

3.11.1 Mutation By Nitrous Acid Exposure

One question that had to be answered before comparing the effect of P-450 expression on mutation frequency was whether the presence of P450IIB1 protein affected the mutation rate of the strain independent of its function as a monooxygenase. To answer this nitrous acid was used as a mutagen. Nitrous acid is a direct acting mutagen requiring no metabolic activation for its effect. The result is shown in Figure 3.17. There appeared to be no significant difference between the two

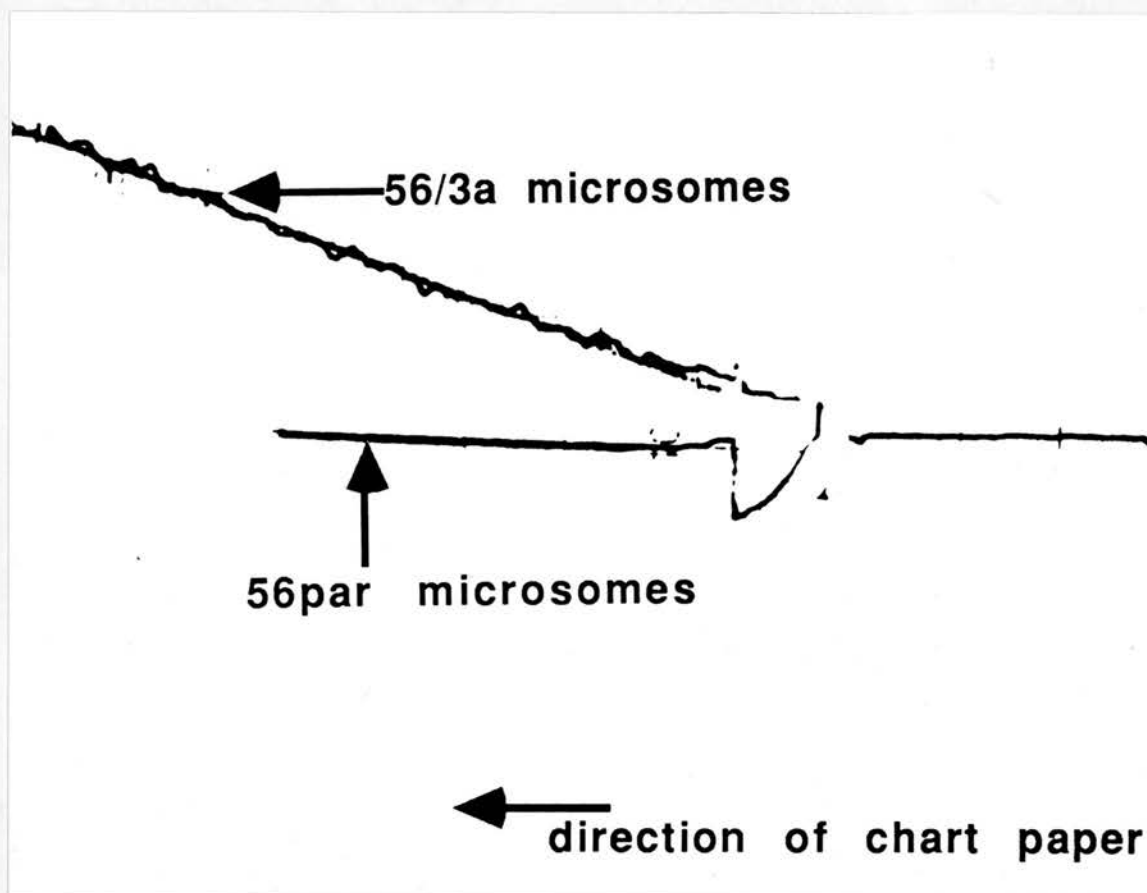


Fig.3.16. Functional Assay on P450IIB1 Protein Expressed in *S.cerevisiae*.

The O-dealkylation of benzyloxyresorufin was carried out according to the method of Burke & Mayer (1974) as modified by Wolf *et al* (1986). The lower line indicates the activity of the p56par microsomes and the upper that of p56/3a. Only the microsomes of the P450IIB1 expressing strain (p56/3a) have significant activity.

strains with the 56/3a strain having a slightly lower mutation frequency. Thus the presence of a foreign protein does not increase the background mutation frequency. It would thus be possible to compare the mutation rates of both the P450IIB1 producing strain and the control strain (56 par) when exposed to a variety of chemicals requiring metabolic activation.

3.11.2 The Use of L-canavanine in Mutation Assays

Kitagawa & Tomiyama (1929) and Kitagawa & Yamada (1932) during studies on urea formation in mammalian liver employed both chemical and enzymatic procedures to study urea levels. The enzymatic procedure using jack bean seed urease gave consistently greater urea values. This led to the discovery of a basic amino acid named canavanine after its isolation from the jack bean, canavalia ensiformis. The occurrence of L-canavanine is limited to the Lotoideae, a major subfamily of the leguminosae (Rosenthal, 1977) where large quantities are stored in seeds, e.g. up to 5% of dry weight in the jack bean seed.

L-canavanine is a structural analogue of L-arginine. The difference being the replacement of the terminal CH₂ group in L-arginine with oxygen. On this basis, L-canavanine can substitute for or antagonize virtually every enzyme controlled reaction for which L-arginine is

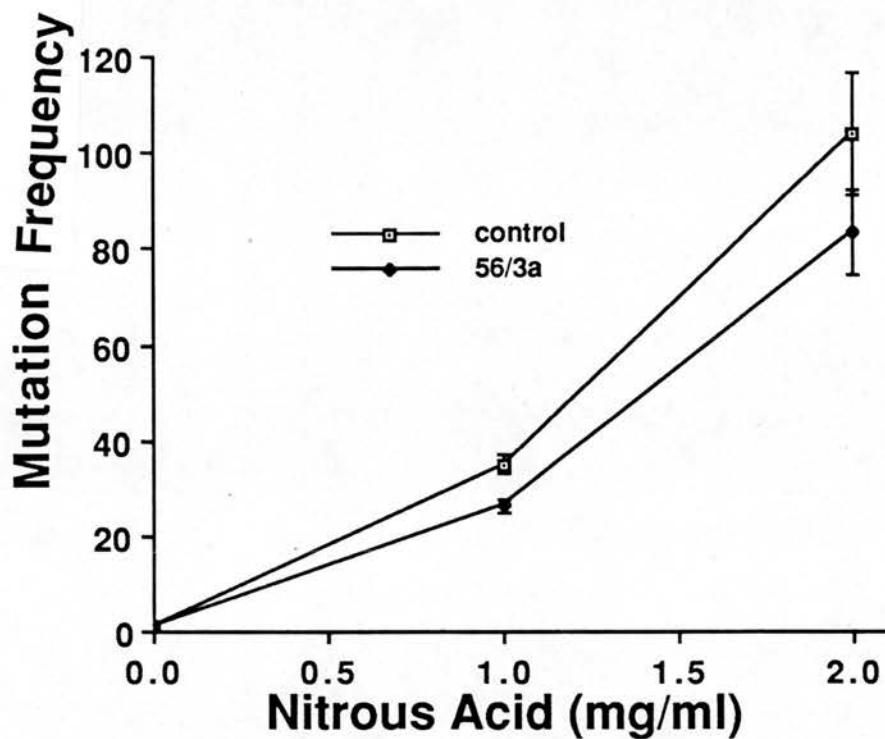


Fig.3.17. Mutation Rate Induced by Nitrous Acid Exposure in Strains p56par and p56/3a.

The direct acting mutagen nitrous acid was used to test the background mutation rates in the two yeast strains (section 2.22). There appeared to be no significant increase in the mutation rate of the P450IIB1 expressing strain (p56/3a) compared to the control strain (p56par). Thus the presence of a foreign protein does not alter the mutation rate of the KY118 yeast strain.

$$\text{Mutation frequency} = \frac{\text{mutations} / 10^6 \text{ survivors}}{\text{background} / 10^6 \text{ survivors}}$$

the preferred substrate. Thus L-canavanine can be activated by arginyl transfer RNA synthetase (Mitra & Mehler, 1967). Once L-canavanine has been linked to the tRNA it can be inserted, in place of L-arginine, into proteins as they are synthesised. Since L-canavanine is chemically much less basic than L-arginine under physiological conditions it is less positively charged than L-arginine (Rosenthal, 1977). This charge difference affects the amino acid interactions determining protein folding thus producing aberrant canavanyl proteins. Evidence (Rosenthal et al, 1987) suggests that proteins structurally altered by the incorporation of L-canavanine are functionally impaired.

Grenson et al (1966) demonstrated that S.cerevisiae actively transports the amino acid L-arginine into the cell using a specific permease and that this uptake can be competitively inhibited by L-canavanine. Furthermore resistance to L-canavanine was shown to be associated with the loss of this arginine permease function. These observations coupled to work carried out by Whelan et al (1979) shows that CAN1 locus codes for the primary structure of the arginine permease.

Whelan et al (1979) also demonstrated the potential of the CAN1 locus in the study of mutation due to the ease of selection for both forward and reverse mutations.

The S.cerevisiae strain KY118 does not have a suitable genetic marker for use in mutation studies. However, the diploid S.cerevisiae series of D4-D7 developed by Zimmerman (1973) for mutational studies do not have the required trp1 genotype to allow the positive selection of p56/3a. It was thus decided to use the development of resistance to L-canavanine as a measure of mutation frequency. The selection for the loss of the arginine permease function means there should be no problem with the type of mutation induced. Any mutation that causes a non-functional arginine permease should be detected.

Compounds belonging to the major classes of chemical carcinogens were tested using the L-canavanine mutation assay as detailed in Section 2.19.2. These were benzo(a)pyrene (a polycyclic aromatic hydrocarbon), sterigmatocystin (a mycotoxin), cyclophosphamide (a nitrogen mustard), 2-anthramine (an aromatic amine), 2-acetylaminofluorene and β -naphthylamine (arylamines). This work was a collaborative project with Ms. Sian Ellard (School of Biological Sciences, University College of Swansea). Each chemical was tested a minimum of three times.

3.11.3 Cyclophosphamide

Cyclophosphamide (CPA) is a widely used chemotherapeutic agent that requires hepatic

bioactivation by P-450 to cause both it's antitumorigenic and mutagenic effects (Brown et al, 1986). CPA is activated to 4-hydroxy CPA which undergoes ring opening to aldophosphamide mustard which spontaneously decomposes to form acrolein and, what is thought to be the biologically active metabolite, phosphoramidate mustard. Since P450IIB1 has been implicated in CPA metabolism (Sladek, 1972; Dearfield et al, 1986), this compound was chosen as a representative of the anticancer drug that requires metabolic activation to exert anti-tumour effects.

A representative dose-response curve obtained when the 56/3a and 56 par strains were exposed to cyclophosphamide is shown in Figure 3.18. Only in the strain expressing the P450IIB1 protein is there a dose-related increase in mutation frequency. This increase was up to 8-fold at 1600 µg/ml CPA relative to that obtained when only DMSO was added to the culture. No such increase in mutation rate was observed in the KY118 strain containing p56 par.

Sladek (1972) has studied CPA metabolism in rats administered with known stimulators and depressors of rat hepatic P-450. The treatment of both males and females with PB increased the rate of CPA metabolism by 7- and 23- fold respectively. Since P450IIB1 is one of the two major PB- inducible proteins in the rat

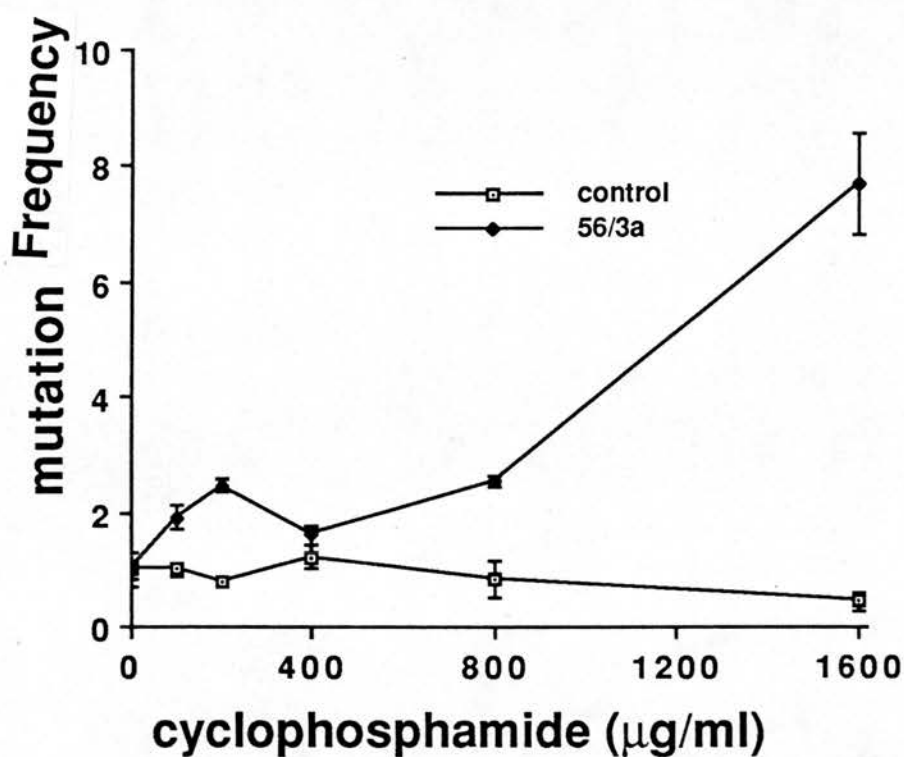


Fig.3.18. Exposure of *S.cerevisiae* Strains to Cyclophosphamide (CPA).

The mutation rate of each strain on exposure to CPA was determined by selection for resistance to L-canavanine (section 2.20.2). The strain producing the P450IIB1 protein (56/3a —◆—) gives a dose related increase in mutation rate (upto 8-fold at 1600 $\mu\text{g/ml}$ CPA) while the control strain (—□—) containing the 56par vector gives no such increase. Mutation rate was calculated as mutations/ 10^6 survivors. This experiment was repeated three times with similar results (maximum mutation rates of 4-; 4-; and 6-fold respectively).

(Adesnik & Atchison, 1986) this suggested a role in CPA metabolism. Dearfield and coworkers have more direct evidence for the role of P450IIB1 using HepG2 cells (Dearfield et al, 1986). These cells were only capable of metabolising CPA when a PB-inducible mRNA was detected using Northern analysis. The probe used, pR17, was prepared from enriched P-450 mRNA from PB-induced rats (Adesnik et al, 1981).

The work discussed above gives strong circumstantial support to the role of P450IIB1 in CPA metabolism, however, the results presented here demonstrate unequivocally that P450IIB1 is involved in CPA metabolism. Also, CPA is not a mutagen in strain KY118 unless P450IIB1 is present. CPA has been shown to be mutagenic in S.cerevisiae (Kelly & Parry, 1983), however, in these experiments endogenous P-450 levels were maximised whereas here endogenous P-450 have been kept to a minimum to decrease background mutation rates. This probably accounts for the lack of induced mutation when the 56 par strain was exposed to CPA.

3.11.4 Sterigmatocystin

Sterigmatocystin (STC) is a carcinogenic mycotoxin produced as a secondary metabolite by Aspergillus, Penicillium and Bipolaris species (Schroeder & Kelton, 1975). In rats, STC has been shown to induce

hepatocellular carcinomas after oral or intraperitoneal administration as well as squamous cell carcinomas after repeated skin applications (Essigmann et al, 1979). STC requires metabolic activation by P-450 and because of its structural similarity to aflatoxin B₁ (AFB₁) is of interest as a model compound for cancer induction. STC is 10-100 times less potent as a hepatocarcinogen in rats than AFB₁ (Dickens et al, 1966).

Figure 3.19 shows a representative curve obtained when the 56/3a and 56 par strains were exposed to STC. As with CPA only the P450IIB1 expressing strain showed a dose related increase in mutation rate on STC exposure. However, the increase was not as pronounced (four-fold relative to the DMSO only control). Again, the 56 par strain showed no equivalent dose-dependent increase in mutation rate.

Work carried out by Essigmann et al (1979) has shown that the active biological intermediate of STC metabolism by P-450 is STC-1,2-oxide. This epoxide binds DNA to give the adduct (N⁷-guanyl -1-hydroxysterigmatocystin). It is proposed that this adduct may account for the toxic effects such as inhibited mitosis and stimulated DNA repair. In these experiments Essigmann used PB-induced rat liver microsomes to activate STC. Again, this suggested a role

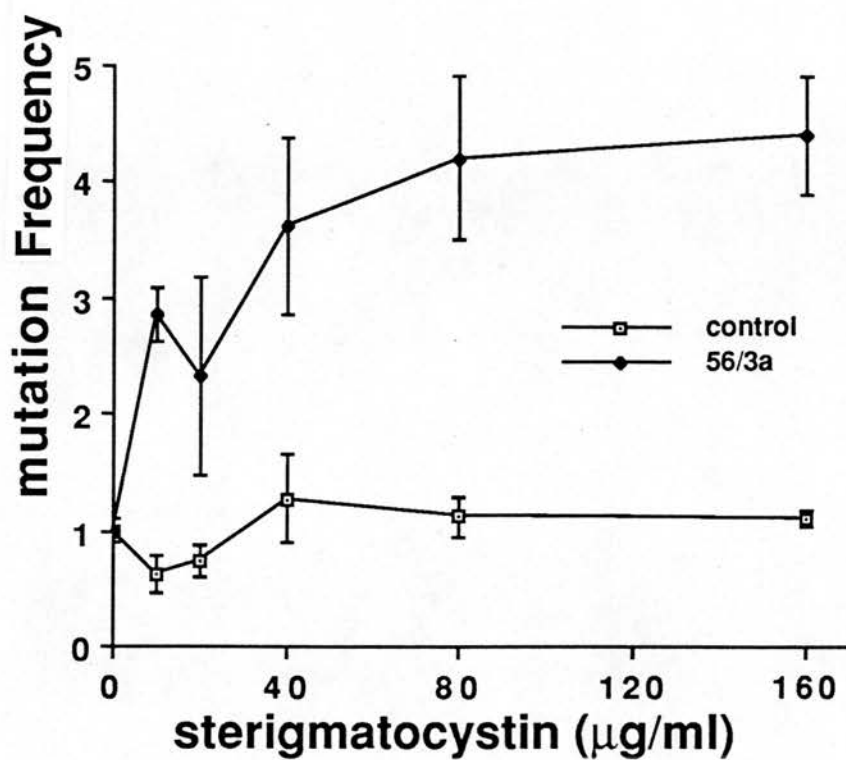


Fig.3.19 Exposure of *S.cerevisiae* Strains to Sterigmatocystin (STC).

The mutation rate on exposure to STC was determined as in Fig.3.18. The P450IIB1 producing strain (—●—) gives an increase in mutation rate of up to 4-fold (at 160µg/ml).The control strain (56par —□—) shows no such increase on STC exposure. This experiment was repeated three times with the same result.

in STC activation for P450IIB1 as the major active PB form in the rat liver. The results (Figure 3.19) support this with a 4-fold increase in mutation frequency occurring at 100µg/ml. This P450IIB1 is involved in STC bioactivation which implicates this protein in the formation of hepatocarcinomas associated with rats administered with STC.

3.11.5 Benzo(a)pyrene

Benzo(a)pyrene (B(a)P) is a polycyclic aromatic hydrocarbon whose metabolic activation by P-450 and epoxidehydrolases gives a dihydrodiol epoxide causing DNA modification (Conney, 1982). 3-Methylcholanthrene (MC)-inducible P-450s are thought to be the major metabolisers of B(a)P while the PB-inducible P-450s are thought not to play an activation role. This is borne out by the result shown in Figure 3.20 where neither the P450IIB1 expressing strain nor the 56 par, control, strain shows any dose related increase in mutation frequency on B(a)P exposure.

Previous work using purified rat liver P-450s (Wilson et al, 1984) has shown that the P450c (P450IA1), the major MC-inducible P-450, has approximately 30 times the activity towards B(a)P than P450IIB1 in a reconstituted system. Thus the expression of P450IIB1 in yeast would not be expected to increase B(a)P associated mutation rates.

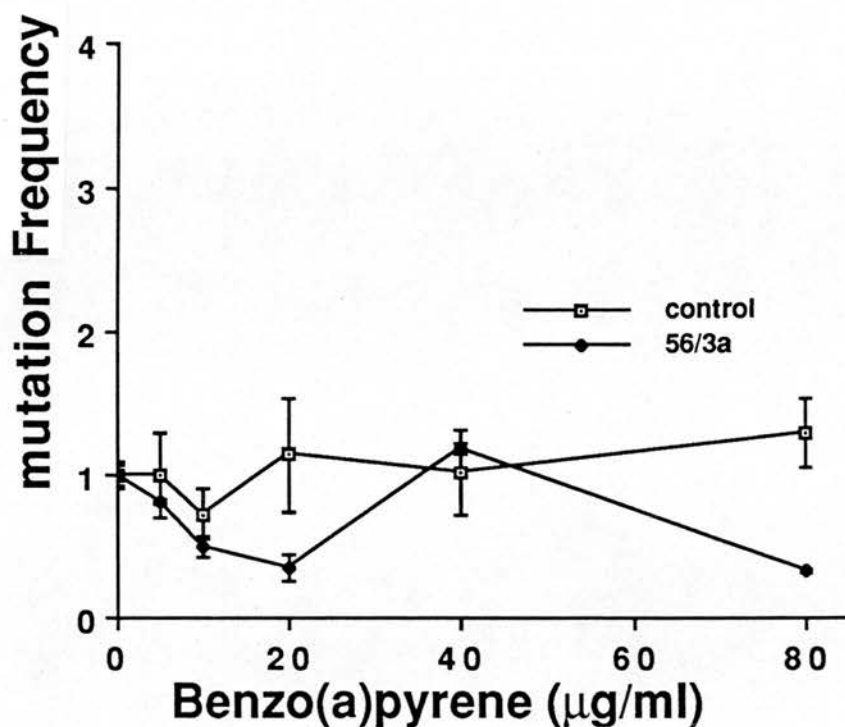


Fig.3.20. Exposure of *S.cerevisiae* Strains to Benzo(a)pyrene (B(a)P).

The mutation rate on exposure to B(a)P was determined as in fig.3.18. Neither the P450IIB1 producing strain (—◆—) nor the control strain (—□—) show any dose related increase in mutation rate when exposed to B(a)P. This experiment was repeated twice with the same result.

The testing of B(a)P in the Ames test (Robertson et al, 1983) has shown that only P-448 (equivalent to P450IA1) will metabolise B(a)P and its 7-8 dihydrodiol derivative to mutagenic products. Again, testing P450-PB (equivalent to P450IIB1) did not result in an increase in the number of trp⁺ revertants. Again this is in agreement with that presented here where P450IIB1 did not cause an increase in mutation rate by the bioactivation of B(a)P.

3.11.6 2-Acetylaminofluorene and β -naphthylamine

These chemicals are both arylamines.

β -naphthylamine (β -NA) exposure has been related to an increase in bladder cancer amongst humans. 2-acetylaminofluorene (2-AAF) was originally proposed as an insecticide before its carcinogenic potential was realised. Arylamines are metabolised by a number of cellular enzymes. They can be acetylated by cytosolic acetyltransferases and then deacetylated by microsomal deacetylases (Hein, 1988).

Arylamines can undergo both N- and C- oxidations to hydroxyarylamines by monooxygenases such as cytochrome P-450 or flavin containing monooxygenases (Kudlubar & Hammons, 1987). The N-hydroxylation is the reaction that produces the active metabolite involved in mutation (Johnson et al, 1980).

A typical mutation experiment for 2-AAF and B-NA is shown in Figure 3.21A and 3.21B. As with B(a)P there was no measurable difference between the 56 par strain and the 56/3a strain for 2-AAF. However, B-NA did show an increase in mutation frequency but this was not dependent on metabolic activation by P450IIB1. The result with 2-AAF is in agreement with the work of Johnson et al (1980) using purified rabbit liver microsomes where the N-hydroxylation of 2-AAF was carried out by forms 4 and 6 (equivalent to rat P450IA2 and P450IA1 respectively) while the P450IIB1 equivalent, form 2, exhibited no catalytic activity with 2-AAF as substrate.

However, work using uninduced rat liver microsomes and antibodies specific to the major proteins of MC- or PB-induced rat livers has shown that a PB-P-450 specific antibody can decrease the mutagenicity of 2-AAF by 30% in an Ames test (Kawajiri et al, 1983). This suggested a role for P450IIB1 in the bioactivation of 2-AAF in uninduced rats. Thus it was hoped that P450IIB1 expression in yeast could definitely answer whether P450IIB1 had a role in 2-AAF activation. The results (Figure 3.21A) support the work suggesting no role for P450IIB1 in the N-hydroxylation (activation) of 2-AAF (Johnson et al, 1980; McManus et al, 1984) rather than that of Kawajiri et al (1983) which suggests it does.

B-NA has also been shown to undergo N-oxidation by rat (equivalent to P450IA1) (Hammons et al, 1985)

Fig.3.21.A

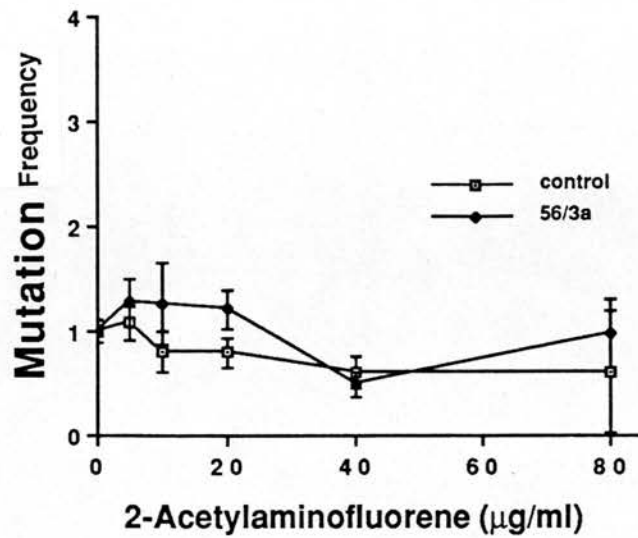


Fig.3.21.B

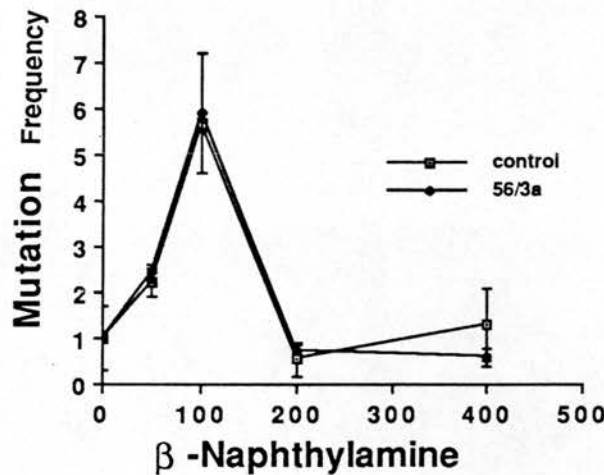


Fig.3.21. Exposure of *S.cerevisiae* Strains to Arylamines.

The P450IIB1 expressing strain (—●—) and the control strain (56par —□—) were exposed to either 2-acetylaminofluorene (2-AAF)(Fig.3.21.A) or β-naphthylamine (β-NA) (Fig.3.21.B) as in fig.3.18. In the case of 2-AAF neither strain gives a dose related increase in mutation rate. For β-NA an increase in mutation rate is seen (up to 100μg/ml) in both strains indicating that the increase is P450IIB1 independent. The decrease in mutation rate at concentrations above 100μg/ml is due to toxic levels of β-NA being reached. These experiments were repeated twice with the same results.

while the P450IIB1 protein was found to have no activity towards β -NA for the N-hydroxylation (activation) reaction although it was found to have 1-hydroxylation (a C-hydroxylation) activity. In this set of experiments β -NA did show a dose-related increase in mutation frequency. However, this was independent of metabolic activation by P450IIB1 since this increase was seen in both the control and P450IIB1 expressing strains. The reason for this apparent non-requirement of bioactivation by P-450 is unknown. Some other monooxygenase within the yeast cell, for example a flavin containing monooxygenase, may activate the β -NA.

3.11.7 2-Anthramine

2-Anthramine (2-AN) is an aromatic amine which is an amino derivative of anthracene. As with 2-AAF and β -NA the activation reaction is an N-hydroxylation. Since the reaction in other aromatic arylamines is carried out preferentially by the MC-inducible P-450 forms would this be true for 2-AN or would P450IIB1 play a role? There was no detectable difference between the two strains in the activation of this compound as seen from Figure 3.22. This is in agreement with recently published work using the Ames test to study 2-AN activation and the effect of specific antibodies to different P-450 isoforms on mutation rate (Lubet *et al*,

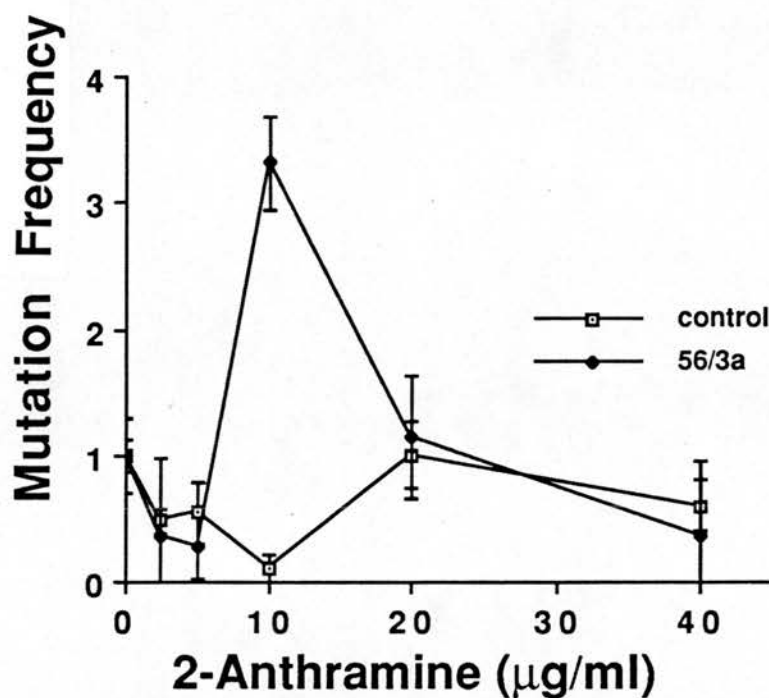


Fig.3.22. Exposure of *S.cerevisiae* Strains to 2-Anthramine (2-AN).

The P450IIB1 expressing (—●—) and the control (—□—) strains were exposed to concentrations of 2-AN up to 40μg/ml as in fig.3.18. The increase in mutation rate observed at 10μg/ml for the strain expressing the P450IIB1 protein was not reproducible when this experiment was repeated.

1989). This work showed that an antibody specific to P450IIB1 (recognising P450IIB2 but no other PB-inducible P-450) did not inhibit trp^+ reversion using aroclor 1254 microsomes as an activation system. The trp^+ reversion could be inhibited by an antibody specific to P450IA1. This implies a role for P450IA1 in the bioactivation of 2-AN but negates a role for P450IIB1. The work here, although it cannot suggest a role for P450IA1 in 2-AN activation, certainly disproves any role for P450IIB1.

3.12 Discussion

It has been proposed (Bridges, 1974) that a rational approach to mutagenicity testing should involve a three-tier strategy. This concept has been updated (Ashby, 1986) such that the first-tier of testing would use in vitro assays to define genotoxins, suggesting that these assays should be the Ames test and the in vitro chromosomal assay according to Scott et al (1983). If a chemical is found to be genotoxic in these assays then the next two stages should be conducted in vivo (if negative all testing should cease and the chemical assigned to be of low genotoxic hazard). The micro nucleus test should be employed for the second tier and if negative then the third tier should be a liver genotoxicity test probably using the unscheduled DNA synthesis (UDS) test. If a negative result is obtained

in both these tests then current evidence suggests that this genotoxin will neither be carcinogenic nor mutagenic to the germ cells of mammals (Ashby, 1986).

However, the best in vitro STT may not be the two described by Ashby (1986) as the requirement for exogenous activation factors limits the efficiency of the assay. Also the NTP program (Tennant et al, 1987) has shown that the concordance of these tests is only approximately 60%. This may be because most human carcinogens require metabolic activation via the P-450 monooxygenase system. Thus it can be imagined that particularly short-lived but highly reactive metabolic species formed outside the cell by these reactions will probably not be able to traverse the various cellular compartments to be in a position to react with DNA and so elicit a detectable response. This would label a chemical as of low genotoxic hazard according to Ashby (1986). Thus an intracellular activation system would be of potential usefulness in an STT.

The results presented here exhibit the potential of genetically engineered S.cerevisiae strains to produce such an STT. Previous work to evaluate the usefulness of S.cerevisiae has shown that although there is potential, there is a problem with narrow specificity and physiological limitations in the endogenous P-450 system. Although protocols have been developed to increase the

levels of yeast P-450 (Kelly & Parry, 1983) and studies have shown that promutagens of relevance to human hazard can be activated (Callen & Philpot, 1978; Parry & Parry, 1988), there is some debate over whether the active species formed are actually those produced in man. However, the use of well defined and easily manipulated yeast expression vectors, as described here, to produce high levels of proteins involved in the bioactivation of promutagens (0.2% of total cellular protein in this case) could be of obvious merit. Not only would the problem of non-detection of highly reactive though unstable intermediates be overcome but the use of a battery of such strains would determine the particular isoenzyme(s) involved in the activation. This is relevant since recent work has shown that there is polymorphic expression of certain P-450 isoenzymes within the population which may have an effect on the predisposition of individuals to cancers with environmental components (Wolf, 1986; Gonzalez et al, 1988).

In this study the major rat PB-inducible P-450, P450IIB1 has been expressed in the S.cerevisiae strain KY118 to produce functionally active protein. The potential of this system to activate promutagens has been evaluated using six chemicals known to be bioactivated. In two cases, cyclophosphamide and sterigmatocystin,

where PB-inducible P-450s have been implicated in the bioactivation reactions the presence of the P450IIB1 protein was shown to increase the mutation frequency in a dose-dependent manner relative to control. In three other cases, 2-acetylaminofluorene, 2-anthramine and benzo(a)pyrene, where P-450s other than the PB-inducible family have been implicated no dose-dependent increase in mutation frequency could be detected. In one case, β -naphthylamine, a dose-dependent increase in mutation frequency was found independent of the presence of P450IIB1 protein. Other monooxygenases such as the flavin containing monooxygenase were probably responsible for this result (Johnson et al, 1980). The last case shows the requirement for adequately controlled STT experiments since without a control mutation rate curve an erroneous conclusion regarding P450IIB1 activation of β -naphthylamine could have been drawn.

Purchase (1982) has proposed that all predicative STT for chemical carcinogenicity should be thoroughly validated using established carcinogens and non-carcinogens. Three stages are proposed:-

- 1) the developing test is subjected to limited validation (using as few as 10 chemicals); 2) a test can be considered developed when adequate validation studies (at least 100 chemicals) have been completed to enable performance criteria to be ascertained; 3) an

established test where large scale validation in several laboratories has occurred. Performance criteria are most commonly based on sensitivity, specificity and predicative value as defined by Cooper et al (1979) such that sensitivity (or true positive fraction) equals the number of carcinogens positive in a test divided by the total number of carcinogens.

Specificity (or true negative fraction) equals the number of non-carcinogens negative in a test divided by the total number of non-carcinogens. Predicative value equals the number of positive results from carcinogens divided by the total number of positive results.

However, the chemicals studied here all are known carcinogens and as such these equations need to be redefined to give an accurate representation of this system thus:

sensitivity equals the number of positive results divided by the number of chemicals activated by PB-inducible P-450 ($2 \div 2 = 1.0$)

specificity equals the number of negative results divided by the total number of chemicals not activated by PB-inducible P-450 ($3 \div 4 = 0.75$)

Predicative value equals the number of positive results from chemicals activated by PB-inducible P-450 divided by the total number of positive results equals two divided by three* which equals 0.67.

* the β -naphthylamine result has been included as a positive result although the increase in mutation frequency was similar in both the control and P450IIB1 expressing strain.

Although the number of chemicals tested was small (six) the predicative value of 0.67 compares favourably to that of the commonly used STT (Tennat et al, 1987). Thus the test described here would seem to have some potential.

However, there are a few flaws that require action before the test could be widely adopted. These flaws are mostly associated with the S.cerevisiae strain used. The mutation rate was determined by the development of resistance to L-canavanine which is due to the loss of arginine permease function (Grenson et al, 1966). This is a general genetic endpoint which will measure any DNA lesion producing a mutation at the CAN1 locus. This has the disadvantage that no information can be obtained on the type of genetic damage a chemical is actually producing. Thus a possible improvement to this test would be the development of strains with genetic markers with specific defects to enable the type of genetic damage a chemical induces (point mutations, small or large deletions etc.) to be determined. Other improvements of the yeast strain could involve the isolation of mutants that are hypersensitive to genetic

damage. For example, by mutating the error-free DNA repair systems in favour of error-prone (SOS-like) pathways. This would increase the sensitivity of the strains to the action of the activated promutagen under test. One could also envisage the mutation of other cellular processes within the cell for example the use of GSH deficient mutants. The thiol compound GSH is known to play a fundamental role in the protection of cells from electrophilic species for example bioactivated cyclophosphamide (Hayes & Wolf, 1988). GSH deficient strains should be more susceptible to genetic damage due to a reduced detoxication capacity. Recent work by Balzi et al (1987) has identified a protein from the PDR1 locus in S.cerevisiae associated with regulation of drug transport. Over expression of this protein is seen to produce hypersensitive cells very susceptible to cytotoxic insult. Thus these type of mutants could also be incorporated into the basic test system detailed here to increase intracellular chemical concentration. This would enable P-450 mediated activation to occur as efficiently as possible.

It has been reported (Ohkawa et al, 1989) that another limitation in mammalian P-450 expression in S.cerevisiae is due to limited production of the endogenous yeast P-450 reductase within the cell. The expression of the rat P-450 reductase in S.cerevisiae has

shown that the protein is highly unstable and thus cannot be used to increase cellular P-450 reductase levels (Ohkawa et al, 1989). Thus a potential method of increasing the activity of a mammalian P-450 in yeast would be to over express the endogenous yeast reductase. The cDNA has been cloned (Ohkawa et al, 1989) and so could be over expressed from a yeast expression plasmid, perhaps via stable integration into the yeast genome using a Yip (Yeast Integrating Plasmid). Alternatively the selection of mutants with increased reductase expression using classical genetics could achieve a similar end.

The potential of the STT described here can, of course, only be developed if a battery of P-450 can be expressed in S.cerevisiae and so act as activation systems for potential promutagens. The work presented here clearly shows that a single P-450 cannot be responsible for the activation of all promutagens even though each P-450 possesses a broad substrate specificity (Nebert & Gonzalez, 1985). Also the P-450 expressed should ideally be from humans, since it is often difficult to determine orthologous and homologous proteins between species, and would better demonstrate human risk.

A similar system to that described here has, in fact, been developed using mammalian cells instead of

S.cerevisiae (Doehmer et al, 1988) where the rat P450IIB1 protein has been expressed in V79 cells. An increase in aflatoxin B₁ (AFB₁) induced mutation associated with P450IIB1 metabolism has been observed. It could be argued that the use of mammalian cells better reflects human risk as cellular processes relate better to those found in the whole animal. However, yeast has the advantage that it is easier to propagate and manipulate. The use of molecular biological techniques to produce exogenous P-450 expressing strains is easier in yeast than in mammalian cells. Also the use of classical genetics to develop strains of yeast which have increased sensitivity to genetic damage or have particular genetic lesions is only possible in S.cerevisiae and not in mammalian cell lines. But whether the undoubted potential of the yeast expression system described here is developed into a widely used STT remains to be seen.

The over expression of mammalian P-450's within S.cerevisiae is a flexible system that can be used for purposes other than as a short-term test system. For example, it could be used to generate large quantities of active metabolites such as the AFB₁-8,9-epoxide or the sterigmatocystin-1,2-oxide which are thought to be the biologically active metabolites of aflatoxin B₁ and sterigmatocystin respectively. The fact that large quantities of protein can be produced from the expression

vectors propagated in S.cerevisiae (up to 1% total protein from the ADC1 promoter) means that the large quantities of protein required for X-ray crystallography or studies on substrate specificity / can be produced from such an expression system. This would reduce the need for complicated isolation protocols required to produce P-450 isoenzymes free from other contaminating P-450. Procedures which take a great deal of time and care when isolating P-450 from mammalian tissue.

Perhaps the most useful application of this expression system is to couple P-450 expression in yeast with site directed mutagenesis (SDM). Here amino acid residues thought to be critical to the function of the protein can be examined and the effect on their substitution on P-450 mediated reactions studied. Furthermore residues thought to be important in the substrate specificity of the protein can also be investigated using the same techniques. These types of study are now starting to be used to answer P-450 structure/function questions (Kimura et al, 1987; Sakaki et al, 1987; Shimizu et al, 1988; Imai et al, 1988; Lindberg & Negishi, 1989).

CHAPTER 4

THE EXPRESSION OF HUMAN GLUTATHIONE S-TRANSFERASES IN *S.CEREVISIAE*

4.1 Resistance to Antineoplastic Therapy

Chemotherapy is an effective treatment for a number of cancers especially when used in conjunction with ionising radiation or surgery. However, in many cases relapses occur because a subset of the tumour cells become resistant to the treatment regimen employed. Frequently resistance to several drugs develops concurrently causing a very serious problem for the patient. Studies in vitro have shown that many genetic changes can be produced by anticancer drug treatment. Some drugs induce point mutations, others chromosome abnormalities or DNA strand breaks, some gene amplification and still others DNA hypomethylation (Warr & Atkinson, 1988). These types of genetic change are partially dependant on the nature of the drug used (Warr & Atkinson, 1988). For example, DNA strand breaks are associated with intercalating agents and drugs such as adriamycin which generate reactive free radicals. Examples of these types of genetic change will be briefly discussed.

4.2 Point Mutations and Drug Resistance

The simplest form of mutation is a base substitution leading to an amino acid change. Evidence that base substitutions or amino acid changes play a role in drug resistance is mainly from work in cell culture (and other

organisms) rather than human tumours. For example, in one study a cDNA for dihydrofolate reductase (DHFR) (the target enzyme for methotrexate) was cloned from a highly methotrexate (MTX) resistant cell line (mouse 3T6) (Simonson & Levinson, 1983). The DNA sequence of the cDNA showed a single base substitution which resulted in an arginine - leucine change at amino acid 22. X-ray crystallography work showed that arginine-22 is a critical residue in the target site for MTX (Simonson & Levinson, 1983). Cells transfected with the mutant DHFR cDNA were highly resistant (Simonson & Levinson, 1983). Thus a simple base change in the correct position within a target protein can result in drug resistance.

4.3 Chromosome Abnormalities and Drug Resistance

Resistance to purine analogues such as 6-mercaptopurine or 6-thioguanine has been shown to involve gross chromosome changes. The target for these compounds is the enzyme, hypoxanthine guanine phosphoribosyl transferase (HGPRT). In one study Fuscoe et al (1983) found that 17 UV-induced purine analogue resistant hamster cell lines contained point mutations within the HGPRT gene. However, another two mutations involved deletions of large sections of the gene (Fuscoe et al, 1983). This finding is important because it emphasises that resistance can be caused by a loss of

gene function, a mechanism which is often overlooked. The most commonly discussed mechanism of resistance being an increase in the levels of a specific protein (or proteins) or mutations which reduce drug sensitivity.

Harris & Collier (1981) have shown that resistance to purine analogues can also be associated with abnormal chromosome numbers suggesting a gene dosage effect in resistance. However, work by Fox & Radacic (1982) has shown that chromosome numbers can return to normal after selective pressure is removed but resistance can remain. One answer to this apparent dichotomy is that a gene duplication has occurred that is undetectable by cytological procedures but is still capable of producing the required gene dosage effects. Low levels of gene amplification such as this has been found in resistance to other drugs such as MTX and 5-fluorouracil (Warr & Atkinson, 1988). The amplification of the DHFR gene during the development of MTX-resistance has been particularly well studied. MTX binds to the DHFR protein with 1000 times higher affinity than the endogenous substrate, dihydrofolate. Toxicity due to MTX can be overcome by overproduction of DHFR. Cell lines have been isolated where DHFR contributes up to 20% of the total soluble protein (Alt *et al*, 1978). Biochemical tests suggest that the DHFR protein produced in the resistant lines is identical to that of their sensitive

progenitors. The increased levels of DHFR protein were shown to be associated with an increase in DHFR gene copy number (Alt et al, 1978). Thus gene amplification can play a role in the development of drug resistance.

4.4 Role of DNA Hypomethylation in Drug Resistance

Mammalian DNA is methylated such that 5-methylcytosine is produced at the sequence CpG. The pattern of methylation is normally clonally inherited and evidence suggests that this can affect gene expression since DNA hypomethylation is thought to be related to gene activation (Bird, 1984). It is interesting that the DNA of benign and malignant human colonic neoplasms have been shown to be hypomethylated (Goelz et al, 1985). Whether this relates hypomethylation to drug resistance in tumours is unclear but is a potential mechanism for the over-expression of drug resistance proteins.

4.5 Multiple Drug Resistance

The previous sections demonstrate that genetic changes can result in multiple drug resistance. The phenotypes of this phenomenon also include alterations in membrane permeability, DNA repair, DNA topoisomerase II and drug detoxification. The roles of each of these will be briefly examined with emphasis on the role of the glutathione S-transferases as drug detoxification proteins in drug resistance.

4.5.1 Changes in Membrane Permeability and Drug Resistance

The development of resistance to certain antineoplastic drugs in mammalian cell lines or in human tumours frequently leads to the onset of resistance to other structurally and functionally dissimilar drugs. This phenomenon has been termed multidrug resistance (mdr). The mdr phenotype has been correlated with changes in the plasma membrane function and structure and associated with a reduced net drug accumulation within the target cell (Gerlach et al, 1986). The mdr phenotype which is associated with a membrane bound drug efflux protein named p-glycoprotein or P-170 can be reversed by the calcium channel blocker verapamil. Verapamil interacts directly with the protein to cause its effect (Gerlach et al, 1986). High levels of P-170 have been detected in some drug resistant human cancers (Bell et al, 1985). Gros et al (1986a) have shown that if a p-glycoprotein cDNA is expressed in mammalian cells they acquire this mdr phenotype. This strongly supports a causal role for this protein in drug resistance mechanism. The sequence of the p-glycoprotein cDNA has yielded much information about its structure and function (Gros et al, 1986b). The protein contains approximately 1,300 amino acid residues and consists of two very similar halves which appear to have arisen as a tandem

duplication (Gros et al, 1986b). Each half of the protein contains a consensus sequence for an ATP binding site which supports the role of the protein as an ATP-dependent drug efflux pump. P-glycoprotein has close homology with a number of bacterial membrane transport proteins (Gros et al, 1986b). The homology is greatest with the E.coli hemolysin transport protein where, over the common region, 60% of the amino acids in the mouse p-glycoprotein are either identical or are conservative substitutions. This degree of homology is one of the greatest ever detected between proteins from pro- and eukaryotes (Warr & Atkinson, 1988). This strong evolutionary conservation indicates that these sequence motifs play a fundamental role in the cell.

It is, however, important to note that each multidrug resistant cell line appears to display a different pattern of cross-resistance and the degree of intracellular drug accumulation does not always correlate with the degree of resistance (Cowan et al, 1986). Changes in other enzymes have also been found in such drug resistant cell lines thus p-glycoprotein is not the sole cause of resistance to anticancer drugs.

4.5.2 Changes in DNA Repair Capacity and Drug Resistance

An increase cellular DNA repair may result in multiple drug resistance by its capacity to reduce DNA

damage. This could limit the usefulness of many antineoplastic drugs especially alkylating agents. However, as with most enzymes potentially involved in *mdr* the precise relationship between altered DNA repair capacity and acquired drug resistance is complicated by the multiple changes occurring within a cell on exposure to a DNA damaging agent. For example, the cellular methylation level may be affected on drug exposure with a subsequent alteration in gene expression.

Correlations have been shown to exist between DNA repair capacity and drug tolerance although this is mainly from work carried out on the O^6 -methyltransferase system in *E.coli*. This enzyme is the product of the *ada* gene and has been shown to have the ability to repair O^6 -methylguanine and other similar lesions (although less efficiently) (Sekiguchi & Nakabeppu, 1987). A similar DNA methyltransferase with high activity towards O^6 -methylguanine is also present in mammalian cells. If this activity is absent (e.g. in CHO cell lines) the cell exhibits hypersensitivity towards alkylating agents such as MNNG. This indicates an important role for this enzyme in the protection of cells from the damage associated with exposure to alkylating agents.

In mammalian cells the repair of *O*-alkylpyrimidines is less well understood than that of *O*-alkylpurines. Although these adducts are repaired the proteins which

appear to be analagous to the E.coli O-alkylpyrimidine methyltransferases do not act on these lesions. This repair pathway must be different to that in E.coli (Brent et al, 1988). Thus techniques are required to further characterise these pathways that do not require the use of E.coli as a model system.

There are other DNA repair processes in mammalian cells that may be involved in drug resistance. These involve excision repair, a process involving the excision of DNA on both sides of an adduct followed by gap removal via the action of DNA polymerases. However, no overall correlation has emerged between the level of excision repair and susceptibility to DNA damage (Fox & Roberts, 1987). Thus more than DNA repair capacity alone may be involved. DNA repair capacity has been shown to be affected by a number of factors. These include the chromatin structure around the DNA lesion with expressing genes preferentially repaired as compared to the genome as a whole . The intracellular level of dNTPs can also affect DNA repair capacity (Fox & Roberts, 1987).

A decreased level of DNA repair also has the potential to produce an mdr phenotype. It has been proposed that the inhibition of DNA replication (by DNA damage) can lead to the reinitiation of replication at a previously unused origin behind the stalled replication fork (Shimke et al, 1986). This could lead to the

amplification of DNA segments whose polypeptide products can confer resistance to chemotherapeutic regimens.

The above suggests that alterations in cellular DNA repair capacity can produce an mdr phenotype. However, the evidence accumulated to date is not conclusive. There are reports that sequences from mammalian genomes have been transfected into hypersensitive mammalian cell lines to produce normal levels of DNA repair (Harris & Hickson, 1989). However, these transfected cell lines are not actually more resistant than the normal wild-type cell line. Thus the study of DNA repair has so far not led to definite answers as to its role in acquired drug resistance.

4.5.3 DNA Topoisomerase II (topII) and Drug Resistance

DNA topoisomerases play an important role within cells by mediating alterations in chromosome structure. Based on their mechanism of action they are divided into two:- type I and type II. Type II enzymes produce breaks in two DNA strands then pass another DNA duplex through the break. The DNA breaks are then repaired. Experiments with yeast temperature dependent topII mutants suggest that this is an essential gene required to unwind daughter chromosomes at the end of mitosis (Di Nardo et al, 1984).

TopII has been used as a target for several

anticancer drugs including VP-16, adriamycin and m-AMSA. These augment the formation of the 'cleavable complex' where topII is covalently attached to the 5'termini of the cleaved double-stranded DNA. The cleavable complex is in some way stabilised so preventing repair of the DNA. This results in the observed cytotoxicity of these topII-active drugs in vivo.

There are four mechanisms by which resistance to topII-active drugs has been shown to occur. Firstly cell lines have been produced that are resistant due to the presence of a mutant topII. For example, VP-16 resistant CHO cell lines produced by ethyl methanesulphonate mutagenesis (Glisson et al, 1986). Identical levels of topII are found in the parental and resistant lines, however, the resistant population contain a heat labile topII that does not form enzyme-DNA cleavable complexes when stimulated by topII-active drugs. Secondly topII levels vary during the cell cycle such that levels are decreased in quiescent cells. This decrease in topII levels can prevent sufficient cleavable-complex being formed and reduce drug cytotoxicity. Thirdly (and less well characterised) topII activity can be regulated in vitro by phosphorylation and poly(ADP)-ribosylation, as well as other factors (Pommier et al, 1986). Fourthly confluent L1210 cells which give equivalent drug-induced DNA damage when compared to logarithmically growing

cells are significantly more resistant to VP-16 induced cytotoxicity (Sullivan et al, 1986). It is, however, unclear why this resistance is observed.

Thus alterations in topII activity has the potential to produce an mdr-phenotype with cross-resistance to several widely used chemotherapeutic agents. It may also represent an attractive target molecule to differentiate normal and neoplastic cells.

4.6 GST-Mediated Detoxification and Drug Resistance

The role of GST in cellular resistance to antineoplastic therapy can be divided into two types:-

- 1) role of GST in intrinsic drug resistance;
- 2) role of GST in acquired drug resistance.

4.6.1 GST and Intrinsic Drug Resistance

Intrinsic resistance to cytotoxic drugs is widely observed in drug therapy. Such resistance is commonly observed in the treatment of parasitic organisms and human tumours. Significant GST levels have been found in human parasites such as nematodes, cestodes and helminths. For example, Kawalek et al (1984) have examined the GST levels in the nematode Haemonchus contortus. The level of GST activity was found to be approximately 2-fold higher in a cambendazole-resistant strain when compared to a sensitive control. This may be

of great significance as nematode resistance is one of the most pressing problems facing antihelmintic chemotherapy. This resistance is on the increase with reports that up to 43% of sheep farms in New South Wales, Australia have drug-resistant strains of nematode (Webb et al, 1979). Other studies have shown that the resistance of mice to Schistosoma infection is due to the recognition of a GST mu-like nematode GST present on the surface of the worm (Smith et al, 1986). The importance of this enzyme in nematode infection and resistance to chemotherapy is unclear. The cestode Moniezia expansa has also been shown the potential to become drug-resistant (Douch & Buchanan, 1978). Thus GST may play a role in the resistance of human parasites to modern chemotherapeutic regimens and thus present a major problem in the fight to eradicate these diseases.

Human tumours have been shown to have high levels of GST prior to chemotherapy and these are found to be intrinsically resistant to chemotherapeutic regimens. For example GST pi has been shown to be expressed at high levels in cancer of the colon, stomach, pancreas and uterine cervix (Kodate et al, 1986; Sato et al, 1987). Immunoblotting has shown GST pi to be overexpressed in adenocarcinoma of the breast and lung, nodular small cell lymphoma and mesothelioma (Shea & Henner, 1987). GST pi has also been shown to be expressed in squamous cell

carcinoma (Hayes & Wolf, 1988). The over-expression of GST pi is also seen in cell lines, for example both small cell and non-small cell carcinoma cell lines (Aswathi et al, 1988; Wolf et al, 1987). It has been suggested that GST pi may be a marker for drug resistance (Ketterer, 1988). However, whether high levels of GST expression represent a significant factor in the failure of chemotherapy remains unclear.

4.6.2 Role of GST in Acquired Drug Resistance

There are four main models that have been studied to evaluate the role of GST in acquired drug resistance. Firstly, the role of GST in the rat hepatocarcinogenesis model (see Farber, 1984). Secondly, the changes in GST expression that occur when normal cells are treated with cytotoxic compounds. Thirdly, changes in GST levels in cell lines resistant to cytotoxic drugs and lastly the examination of GST in tumours before and after the onset of resistance to antineoplastic therapy. The changes which occur in GST subunit expression within these models are discussed overleaf.

4.6.3 GST Expression in Rat Hepatocarcinogenesis

The changes that are induced within the rat liver by feeding a hepatocarcinogen, such as aflatoxin B₁ represent the most studied in vivo model which

implicates the GST in drug resistance (Hayes & Wolf, 1988). The importance of drug resistance in the development of preneoplastic foci to liver tumours has been emphasised by Farber (1984). The initial response in the rat liver when exposed to a hepatocarcinogen is an overall increase in alpha and mu class enzymes (specifically subunits 1 & 3) (Kitahara et al, 1984). Subsequently, subunits belonging to all three types of GST become over-expressed in pre-neoplastic foci. These subunits are 1, 3 and 7 (from the alpha, mu and pi class GST respectively) (Sato et al, 1984).

Many of these pre-neoplastic foci develop into nodules rich in GST isoenzymes and eventually into neoplastic tumours. However, most redifferentiate into apparently normal hepatocytes (Farber, 1984). Initially the hepatomas resulting from the nodules have the same overall expression of subunits 1, 3 and 7 as in the nodules. As the tumour progresses all but subunit 7 (pi class enzyme) is repressed (Ketterer, 1988). It has been proposed that subunit 7 can be used as a biochemical marker for the development of hepatocarcinogenesis.

The role of GST in the progression of pre-neoplastic foci to fully fledged primary hepatomas has not as yet been determined. However, since drug resistance has been proposed as an important link in these events (Farber, 1984) the over-expression of GST

may give the pre-neoplastic foci a growth and hence selective advantage over the neighbouring cells. Indeed these cells have been shown to be drug resistant. The over-expression of GST in neoplasia may not be an important part in the process, but may be an indirect consequence of the changes which occur. For example, the 5'upstream region of the GST pi promoter contains a phorbol ester responsive element, TRE (Sakai & Muramatsu, 1987) which also appears to be activated by the c-Ha ras protooncogene (Sakai et al, 1988). Thus the expression of GST pi may be a consequence of c-Ha ras expression within the pre-neoplastic foci and nodules (Cowell et al, 1988).

4.6.4 GST Changes Induced in Normal Cells by Cytotoxic Drugs

The bone marrow in male CBA mice has been used as a model to investigate the response of normal cells to the cytotoxic effects of cyclophosphamide, 1- β -D-arabinofuranosylcytosine (araC) and X-irradiation (Adams et al, 1985). The bone marrow is of interest due to the toxicity caused in this tissue by the anticancer drugs used in modern chemotherapy. Indeed the bone marrow is often the origin of secondary cancers caused by treatment with chemotherapeutic agents. As mentioned previously (Section 1.10.4) exposure of an animal to a

low dose of a cytotoxic agent has the potential to protect against a normally lethal dose of the same compound (Millar et al, 1975). Thus a low dose of cyclophosphamide was used (75 mg/kg) to 'prime' the bone marrow. This was found to cause an initial decrease in GSH levels (Adams et al, 1985). However, 5-7 days post-treatment the GSH and GST levels were found to be enhanced (2-3 fold in both cases). Animals given an otherwise lethal dose of cyclophosphamide (350 mg/kg) during this period were found to survive (Adams et al, 1985).

Similar effects were seen with araC and X-irradiation. The increase in GSH and GST induced by the different compounds may thus represent a general "stress" response of cells to cytotoxic injury.

4.6.5 Role of GST in Resistance within Human Tumours

In an in vivo study of drug resistance two ovarian cell lines were derived from the ascites of a patient before (PE01) and after (PE04) the development of clinical drug resistance to cisplatin, chlorambucil and 5-fluorouracil (Wolf et al, 1987a). The PE04 cell line was shown to be 3-fold more resistant to cisplatin and chlorambucil but not more resistant to 5-fluorouracil (Wolf et al, 1987a). The glutathione and glutathione-dependent enzymes of these cell lines have also been

examined and differences have been observed. It was found that GSH levels were increased 1.4-fold in PEO4 while γ -glutamyltranspeptidase, an enzyme involved in the regulation of GSH synthesis (Ahmad et al, 1987), was elevated 6.3-fold (Lewis et al, 1988a). Overall, an increase in GST levels of 2.9-fold were measured. Activity towards both ethacrynic acid and CDNB were increased (Lewis et al, 1988a). This difference could not be associated with the expression of a specific GST subunit as both pi and alpha class enzymes could be detected at similar levels. The mu subunit was absent from both. However, this study provided the first evidence that tumour cells obtained before and after the onset of drug resistance have differences in GSH and GSH-dependent enzymes. It is highly supportive of a role for GST and GSH in acquired drug resistance.

4.6.6 Role of GST in The Resistance of Mammalian Cell

Lines to Cytotoxic Drugs

There are examples of all three classes (alpha, mu and pi) of cytosolic GST being over-expressed in cell lines made resistant to cytotoxic drugs. Also, Clapper & Tew (1989) have reported a role for a 29kD microsomal GST in acquired resistance to nitrogen mustards in a Walker 256 rat mammary carcinoma cell line. However, as this chapter deals with the human cytosolic GST these will

be given greater emphasis.

An MCF-7 human breast cell line has been selected for resistance to adriamycin (Cowan et al, 1986; Batist et al, 1986). A 45-fold increase in GST activity was measured using CDNB as substrate. This was associated with the appearance of a pi class enzyme, pi. The resistant cell line, although selected for resistance to adriamycin, was also shown to be resistant to the vinca alkaloids (vincristine and vinblastine), VP-16 and actinomycin D, i.e. a classic mdr phenotype (Cowan et al, 1986). The resistance was greatest towards vinblastine (375-fold). It should also be noted that an increase in GST pi expression is not the only biochemical change associated with the MCF7 Adr^R cell line (Cowan et al, 1986). Other biochemical changes measured include an overall decrease in intracellular levels of adriamycin (2-3-fold) associated with an increase in p-glycoprotein activity (Batist et al, 1986). Also, there is a decrease in Cytochrome P-450 levels (Phase I - toxification enzymes), as estimated by arylhydrocarbon hydroxylase activity, of at least 40-fold (Cowan et al, 1986). Other phase II enzymes are also increased, the UDP-glucouronyltransferase enzymes are increased 2-3-fold with respect to the wild-type MCF-7 cell line (Cowan et al, 1986). It is interesting that the changes observed in this cell line mirrored those found in rat

hyperplastic nodules. In view of the range of biochemical changes induced by adriamycin exposure it is difficult to completely assign a role for GST pi in the acquired resistance observed. It may be that the GST pi is merely a marker for resistance. The alpha class of GST enzymes have also been implicated in drug resistance by the work of Robson et al (1987). A chinese hamster ovary (CHO) cell line was produced that was resistant to chlorambucil (Robson et al, 1986) and designated CHO-Chl^r. The total GST activity in the resistant cell line was increased 3-fold (as measured by CDBB activity) while a 2- and 5-fold increase in GST-mediated ethacrynic acid conjugation and peroxidase activity towards cumene hydroperoxide were also measured (Robson et al, 1987). Consistent with these changes were an increase in GST pi and alpha class enzymes (Robson et al, 1987). No increase in p-glycoprotein could be detected, however, GSH levels were increased (2-fold) (Robson et al, 1987). It is thus entirely possible that the resistance to chlorambucil may be mediated by the increase in intracellular thiol concentration rather than the increase in GST activity. GSH has been implicated in the detoxification of nitrogen mustards including chlorambucil (Wolf et al, 1987b).

The mu class of enzymes have been implicated in cellular resistance to 1,3-bis (2-chloroethyl)-1-

nitrosourea (BCNU) by the recent work of Smith et al (1989). A 9L rat gliosarcoma cell line has been derived (9L-2) that is 3-4-fold more resistant than the parental cell line (Smith et al, 1989). Immunoblotting showed that the 9L-2 subline, which had an overall decrease in GST activity, had elevated levels of the mu class enzymes. The same analysis showed a decrease in pi class GST levels in 9L-2. In support of this finding the mu class GST were shown to have the highest activity in the deactivation of BCNU. A Vmax to Km ratio of 29.2 for 3-4 and 26.1 for 4-4 respectively were measured. The alpha class enzyme 1-2 had a ratio of only 2.3 while the pi enzyme, 7-7, also had very low activity towards BCNU as substrate (Smith et al, 1989). This study provides strong evidence for the role of the over-expressed GST in drug resistance. It is one of the few studies to have shown that the drug used to produce the resistant subline is in fact a GST substrate. Dulik et al (1986) have produced the only other study demonstrating that GST are directly involved in the detoxification of anticancer drugs. Melphalan was shown to form GSH conjugates in the presence of GST. Both microsomal and cytosolic GST were shown to be involved (Dulik et al, 1986; Dulik & Fenselau, 1987). The rate of conjugate formation was increased upto 100-fold in the presence of GST (Dulik et al, 1986).

Thus, all three classes of GST have been implicated in the resistance of cell lines to cytotoxic drugs. However, the evidence for the involvement in the resistance mechanism is circumstantial. It is also not clear whether GST over-expression is causal or coincidental in cell lines resistant to anticancer drugs. The expression of cDNAs encoding human alpha and pi class GST in S.cerevisiae, was carried out here to try and resolve the situation.

RESULTS

4.7 Subcloning of Human Alpha Class GST into pMA56

The isolation of the alpha class GST (B₁B₁) used in these studies has been reported previously (Lewis et al, 1988b). Initially a large scale plasmid preparation was carried out on the plasmid designated pUC/B₁. An EcoRI restriction digest followed by preparative agarose gel electrophoresis was used to show that the plasmid (pUC/B₁) did indeed contain the human alpha class GST.

Following an EcoRI digestion of pUC/B₁ DNA (20µg) the GST alpha cDNA was isolated by passage through a spin-X column. The cDNA was then ligated into the p56-RIBAP vector (see Section 3.3). After transformation into E.coli DH1 the resultant colonies were subjected to the colony screening method of Grunstein and Hogness (1975) using ³²P-labelled GST B₁

cdna as a probe. This gave six possible colonies containing recombinant plasmids (Figure 4.01). Plasmid preparations were carried out on these colonies and restriction analysis with EcoRI used to determine which contained the GST B₁ cdna as insert. This is shown in Figure 4.02. The plasmids designated 56/B₁-1; 56/B₁-3 and 56/B₁-6 were shown to have cdna inserts. Each of these were transformed into the S.cerevisiae strain KY118 to determine which produced GST B₁B₁ protein.

4.8 Expression of GST B₁B₁ Protein in S.cerevisiae Strain KY118

A single colony from each transformant was inoculated into supplemented synthetic SD medium (50 ml) and grown into stationary phase. Cells were harvested and cytosolic preparations (Section 2.16.3) subjected to SDS-page and Western blotting. Antibodies raised against the purified human alpha class GST (B₁B₁) were used followed by ¹²⁵I-labelled protein A to give the result in Figure 4.03. Both the p56/B₁-1 and p56/B₁-6 plasmids gave bands which comigrated with the purified human GST B₁B₁ standard. The p56/B₁-6 containing strain was used in subsequent analyses as it gave a slightly stronger cross-reacting band.

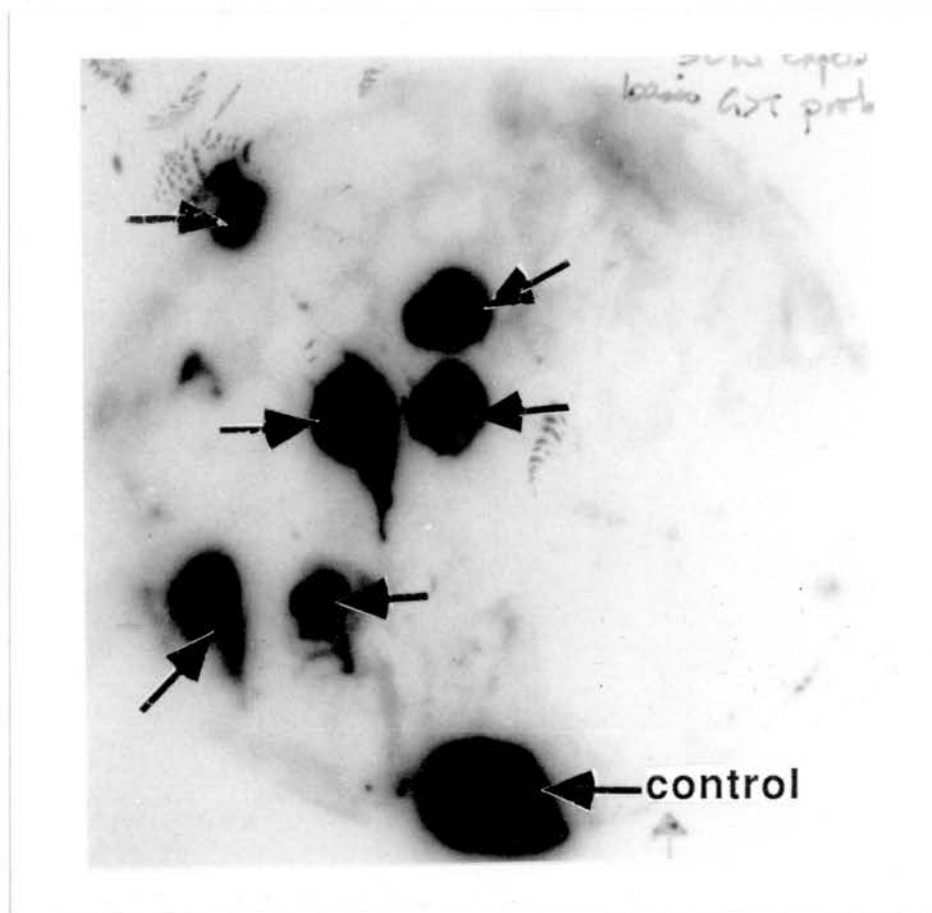


Fig.4.01. Colony Hybridisation Analysis on Transformed E.coli DH1.

The colony hybridisation method of Grunstein & Hogness(1975)(section 2.15) was used to determine if the p56/B₁ vector was present in the transformed E.coli DH1 colonies. ³²P-labelled human alpha class cDNA was used as a probe giving six possible p56/B₁ carrying colonies (marked by arrow). The control is E.coli JM101 transformed with the pUC/B₁ vector.

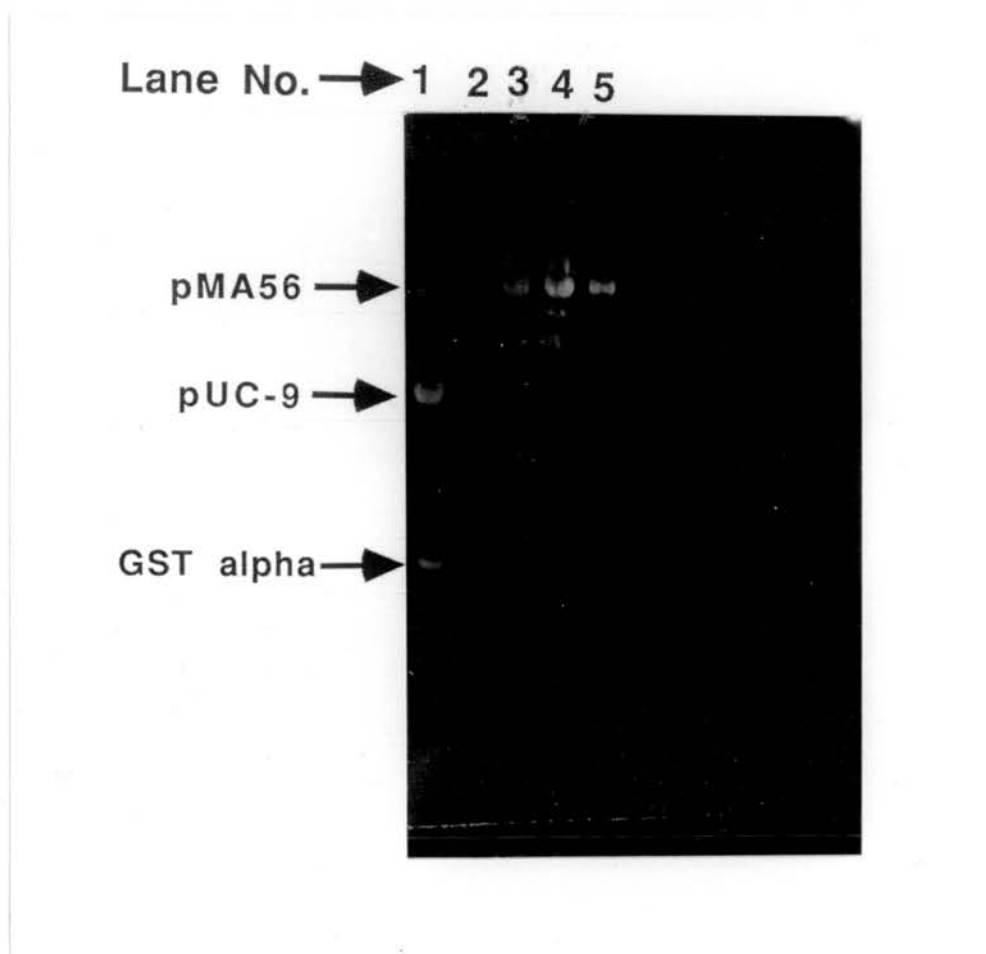


Fig.4.02. Restriction Analysis on Possible p56/B₁ Clones.

EcoRI restricted 56/B₁ DNA (1μg) isolated from E.coli DH1 transformants was loaded in lanes 2-4 (corresponding to 56/B₁-1, 56/B₁-3 and 56/B₁-6 respectively). EcoRI digested pUC/B₁ DNA (1μg) was loaded in track 1 and EcoRI restricted pMA56 DNA in track 5. All three EcoRI digested 56/B₁ plasmids were found to have a cDNA insert co-migrating with that of pUC/B₁.

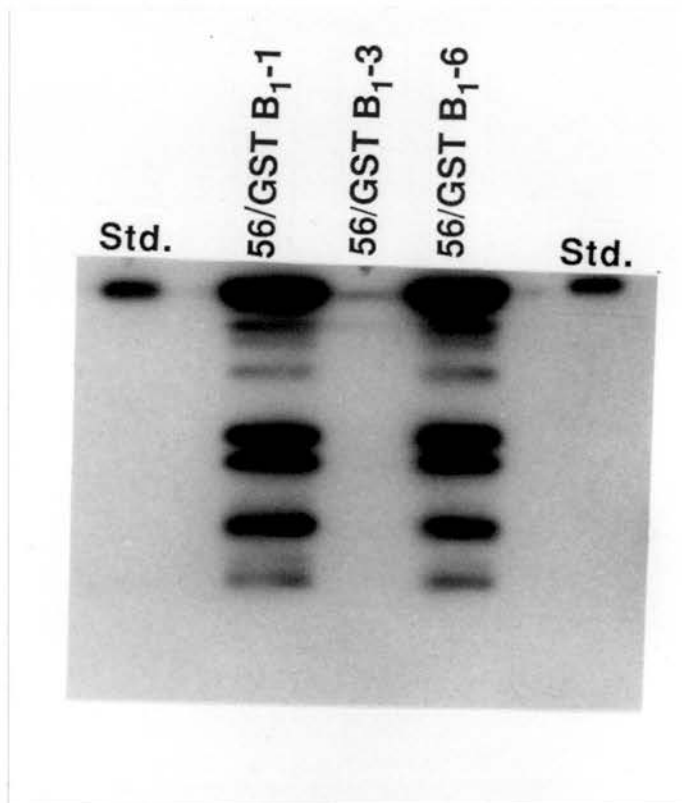


Fig.4.03. Expression of Human Alpha Class GST in S. cerevisiae.

SDS-page followed by Western blot analysis (section 2.19) was carried out on the cytosolic fractions (50µg) of p56/B₁-1, p56/B₁-3 and p56/B₁-6 transformed KY118. Antibodies raised against the human alpha class GST (B₁B₁) (Stockman *et al*,1987) were used to identify the presence of the human GST protein. Purified human alpha class GST protein (B₁B₁) was loaded as standard. A band co-migrating with the alpha class GST standard was found in the p56/B₁-1 and p56/B₁-6 transformed strains. The lower molecular weight bands are due to the degradation of the B₁B₁ protein.

The cytosolic fractions were prepared as detailed in section 2.16.3.

4.9 Subcloning of Human Pi Class GST into pMA56

The human pi class cDNA was a generous gift from Professor M. Muramatsu (Dept. Biochemistry, Tokyo, Japan). Its isolation has been detailed elsewhere (Kano et al, 1987). Briefly the rat GST-P cDNA was used as a probe to screen a human placenta cDNA library in λ gt11. The cDNA was isolated from purified phage clones by EcoRI digestion. The cDNA was subcloned into the EcoRI site of pUC-19. The cDNA has been sequenced and shown to be full-length (Kano et al, 1987) consisting of 630bp coding sequence and a 78bp non-coding 3'-region. The cDNA was isolated as in Section 4.7 and ligated to p56-RIBAP (Section 3.3). The Grunstein & Hogness colony screening procedure (1975) was used to screen the transformed E.coli DH1. This gave a possible 27 recombinants (Figure 4.04). Six colonies were taken for plasmid preparations. The plasmids obtained designated 56/Ac-1 to 56/Ac-6 were digested with EcoRI and subjected to preparative agarose gel electrophoresis (Figure 4.05). Of the six plasmids only p56/Ac-5 did not possess a cDNA insert. Each of the plasmids containing the GST pi cDNA were transformed into S.cerevisiae strain KY118 as Itoh et al (1983).

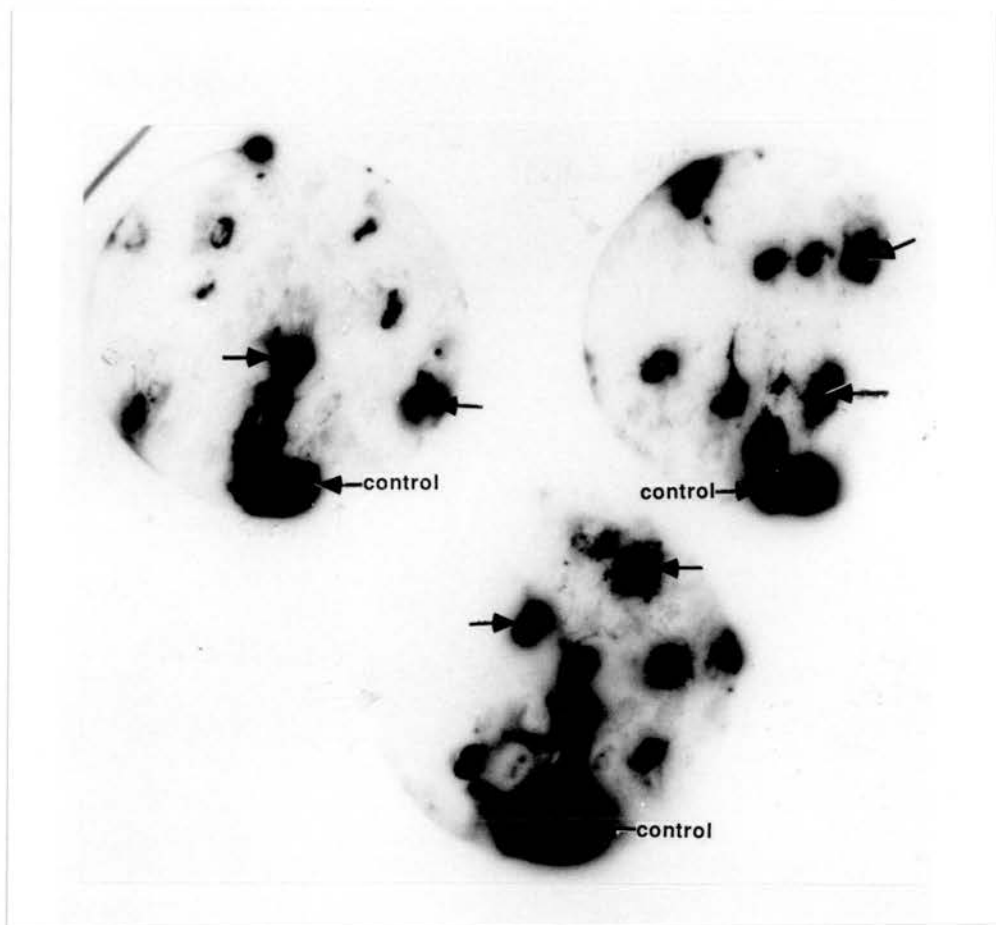


Fig.4.04. Colony Hybridisation Analysis on Transformed E.coli DH1.

The colony hybridisation method of Grunstein & Hogness(1975) was used to determine if the p56/Ac vector was present in the transformed E.coli DH1. ^{32}P -labelled pi class GST cDNA was used as a probe. This gave 27 possible p56/Ac containing colonies from which six were picked at random (marked by arrow). The control is E.coli JM101 transformed with the pUC/Ac vector.

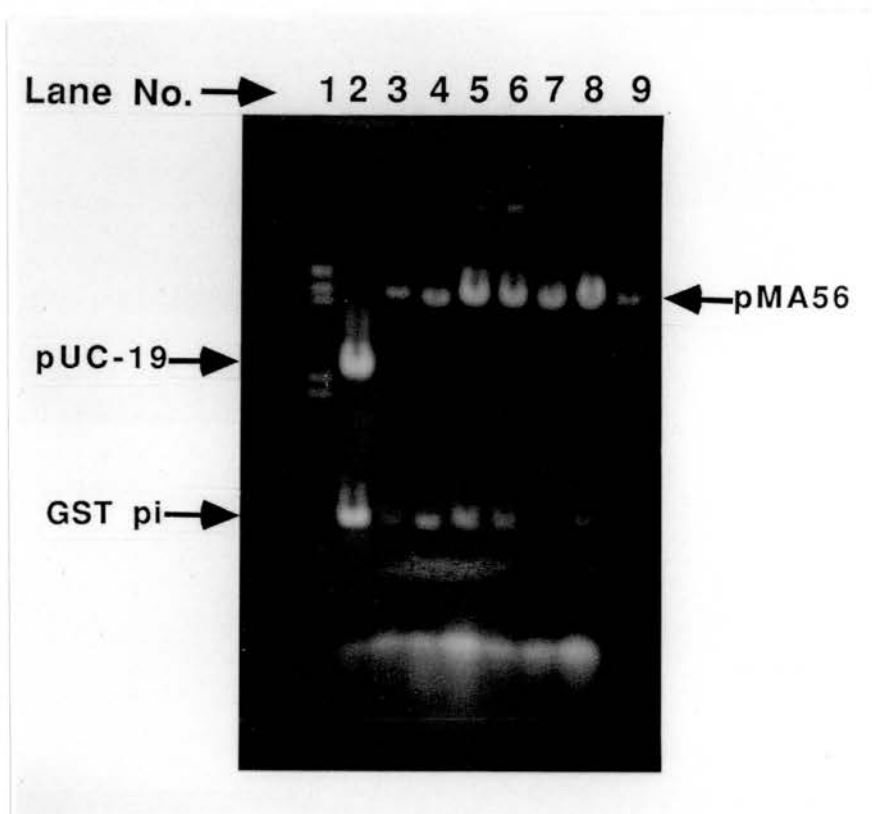


Fig.4.05. Restriction Analysis on Possible p56/Ac Clones.

EcoRI restricted 56/Ac DNA (1 μ g) from E.coli DH1 transformants was loaded in lanes 3-8 (corresponding to 56/Ac-1-6 respectively). Hind III digested phage λ DNA (1 μ g) was loaded in lane 1. In lanes 2 and 9 was loaded EcoRI digested pUC/Ac and pMA56 DNA respectively. Only the plasmid designated p56/Ac-5 lacks the cDNA insert co-migrating with that of pUC/Ac.

4.10 Expression of GST Pi Protein in S.cerevisiae Strain KY118

Only the plasmids designated 56/Ac-2; 56/Ac-3; 56/Ac-4 and 56/Ac-6 gave colonies on transformation. These colonies were inoculated into supplemented synthetic SD medium (50ml), harvested and cytosolic preparations subjected to SDS-PAGE and Western blotting using antibodies raised against purified human GST pi protein and ^{125}I -labelled protein A (Figure 4.06). Only the strain containing the 56/Ac-4 plasmid produced a protein band comigrating with purified pi GST standard. This strain was used in subsequent experiments.

4.11 Subcellular Localisation of GST Proteins

Subcellular fractions were prepared from the yeast strains transformed with the p56/B₁-6; p56/Ac-4 and p56/Ac-3 (as control) vectors using the method detailed in Section 2.16.1. These fractions were subjected to SDS-page and Western blotting using anti-GST pi and anti-GST alpha antisera (Figure 4.07). As expected the GST proteins were localised in the cytosolic fraction with little or no protein being present in the microsomal (endoplasmic reticulum) fraction. Densitometric analysis and comparison with GST B₁B₁ or GST pi standards indicated that both the GST pi and GST B₁B₁ proteins were produced at 0.5% of total yeast protein.

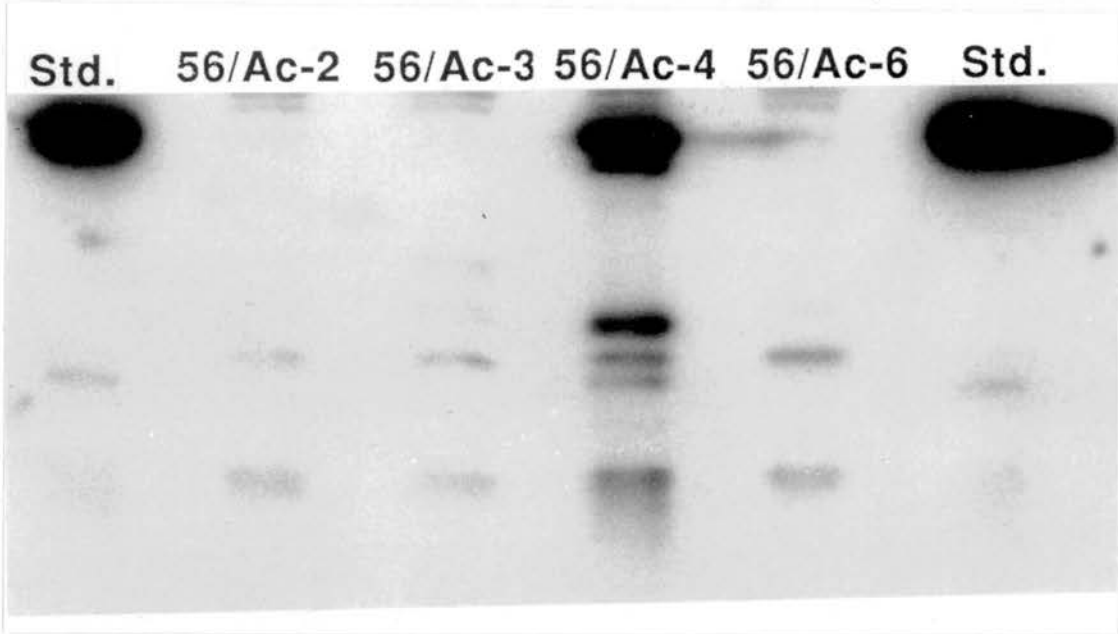


Fig.4.06. Expression of Human Pi Class GST in S. cerevisiae.

SDS-page followed by Western blot analysis was carried out on the cytosolic fractions(50 μ g) of p56/Ac-2, p56/Ac-3, p56/Ac-4, and p56/Ac-6 transformed KY118. The standard corresponds to purified human pi class GST protein. Only the p56/Ac-4 transformed strain produces a band which co-migrates with the standard. The cytosolic fractions were prepared as detailed in section 2.16.3.

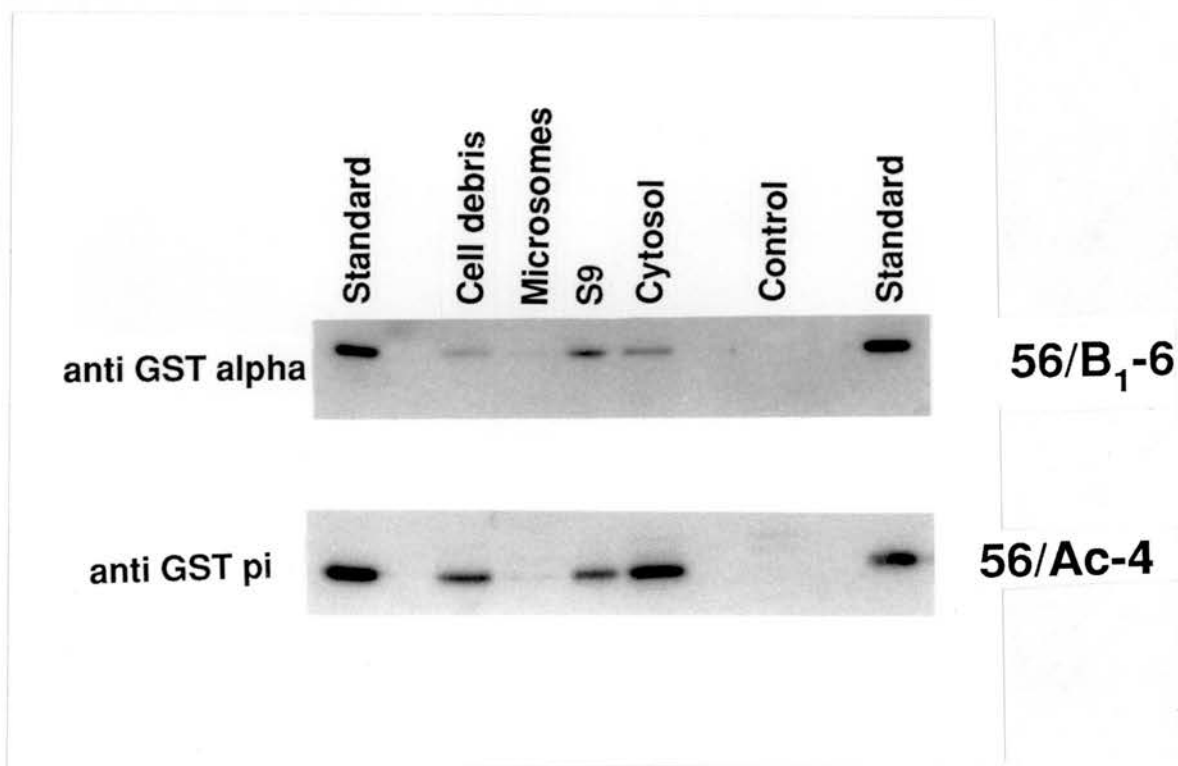


Fig.4.07. Subcellular Localisation of Human GST Proteins in S.cerevisiae.

Protein samples (25µg) from various yeast subcellular fractions were separated by SDS-page followed by Western blot analysis using antibodies to GST pi and GST B₁B₁. Subcellular fractions were prepared as described in section 2.16.1. Cell debris = 10,000g pellet; S9 = 15,000g supernatant; microsomes = 246,000g pellet; cytosol=246,000g supernatant. Standards were either purified human GST alpha (B₁B₁) or pi protein. No cross reacting bands were observed in the cytosol of the yeast containing the pi class GST cDNA inserted into the pMA56 vector in the reverse orientation (control).

4.12 Functional Activity of Expressed GST Proteins

The activity of the expressed human GST proteins was initially determined using the general GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). Yeast strains expressing either the human alpha or pi class GST have high activity in the conjugation of CDBN. The activity of the pure alpha class GST towards this substrate is in the order of 64 $\mu\text{mol}/\text{min}/\text{mg}$ protein with pi being 105 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Mannervik & Danielson, 1988). The activities determined using CDBN are shown in Figure 4.08. The control strain had an activity towards CDBN as substrate of less than 10 $\text{nmol}/\text{min}/\text{mg}$ cytosolic protein (10 units). The GST pi producing strain had activity of up to 1000 units with a normal range between 350-600 units. Maximal activities for the alpha class GST producing strain were also up to 1000 units. However, the usual level was between 100-250 units. Thus both GST were active towards CDBN when expressed in S.cerevisiae. This activity was usually 10-50-fold higher than in the control culture the activities being of a similar level to those found in human liver cytosol.

The large variation in activities measured may be due to the time in the growth phase where the cells were harvested. It was interesting that cultures harvested in stationary or early logarithmic phase had less GST activity than those harvested in mid to late logarithmic

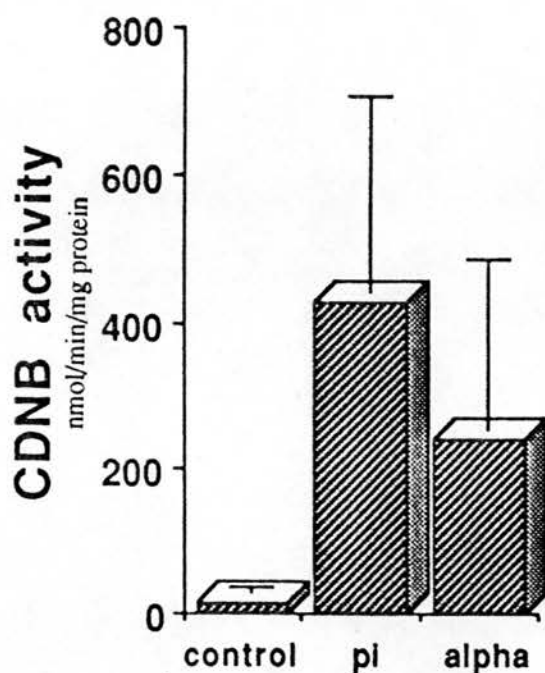


Fig.4.08. GST Activity Towards 1-Chloro-2,4-Dinitrobenzene (CDNB) in Cytosolic Fractions of *S.cerevisiae*.

The GST activity of the yeast strains 56/Ac-3 (control); 56/Ac-4 (GST pi) and 56/B₁-6 (GST alpha) was measured using the GST-mediated conjugation of CDNB with GSH at 30°C according to the method of Habig *et al* (1974) (section 2.17.5). The data represents the mean \pm standard deviation for twenty two determinations and is expressed as nmol/min/mg cytosolic protein.

growth. Whether this is due to increased protein accumulation during cellular growth or an increased rate of degradation in stationary phase is unknown. The pi class GST appeared to be more stable within the stationary yeast cultures than the alpha class protein. The alpha class GST appeared to be degraded within the cell as the cultures became stationary. CDNB activity was also reduced in stationary phase. It was found that 4 hours after inoculation into fresh medium GST B₁B₁ activity was only 58 units compared to a control value of 11 units while the GST pi activity was already 300 units.

Cumene hydroperoxide was also used to test for GST activity. Only the human alpha class GST has peroxidase activity towards this substrate. The specific activities of the purified alpha and pi class proteins are 10.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein and pi = 0.03 $\mu\text{mol}/\text{min}/\text{mg}$ protein respectively (Mannervik & Danielson, 1988). As predicted the control and pi expressing strains had essentially the same level of peroxidase activity (5 units) whereas the alpha expressing strain had a level of up to 100 units (Figure 4.09).

4.13 Measurement of Glutathione (GSH) Levels in The Yeast Strains

It has been shown that GSH is involved in the detoxification of two types of anticancer drugs,

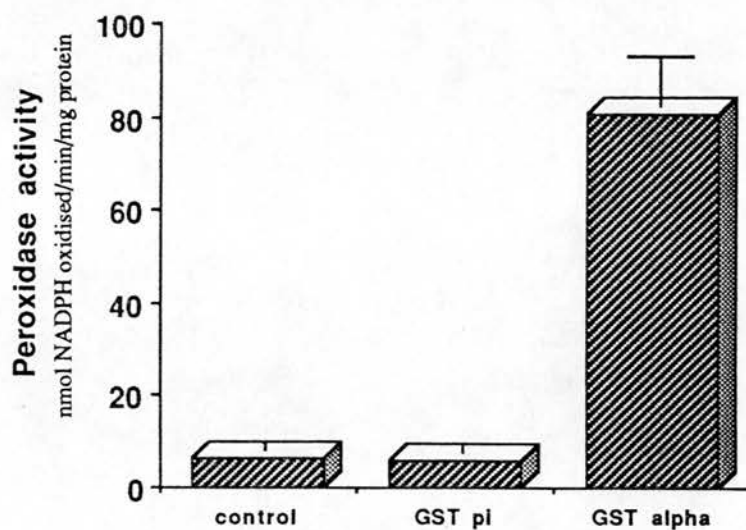


Fig.4.09. GST Activity Towards Cumene Hydroperoxide (CHP) in Cytosolic Fractions of S.cerevisiae.

The peroxidase activity of the yeast strains 56/Ac-3 (control), 56/Ac-4 (GST pi) and 56/B₁-6 (GST alpha) was estimated using CHP in a coupled reaction by measuring the rate of NADPH oxidation (section 2.17.6B). The data represents the mean \pm standard deviation for six determinations.

electrophilic agents (melphalan, cyclophosphamide, chlorambucil) and reagents generating reactive oxygen (adriamycin, daunorubicin, mitomycin C) (Wolf et al, 1987b). Various studies have shown a relationship between GSH level and tumour cell resistance (Calcutt & Connors, 1963; Connors, 1966; Lewis et al, 1988a). It was, therefore, important to establish whether the over-expression of the GST altered cellular GSH levels. The mean values of GSH were calculated as 24.9 ± 2.9 , 23.0 ± 6.9 , 25.8 ± 9.7 nmol GSH/mg soluble protein for the control, GST alpha and GST pi expressing strains respectively (Figure 4.10). Thus a variation in GSH could not play a role in the differential sensitivity of the three strains when exposed to cytotoxic drugs.

Now that it had been clearly established that GST could be expressed in S.cerevisiae in a functional form it was of central importance to establish whether these expressing strains had altered resistance to anticancer drugs. A description of these experiments and a brief description of the mechanism of action of the compounds used is given below.

4.14 Cytotoxicity Assays on Yeast Strains

4.14.1 Effect of GST Expression on Adriamycin Toxicity

Adriamycin is a member of the anthracycline antibiotics which had their first clinical trials in the

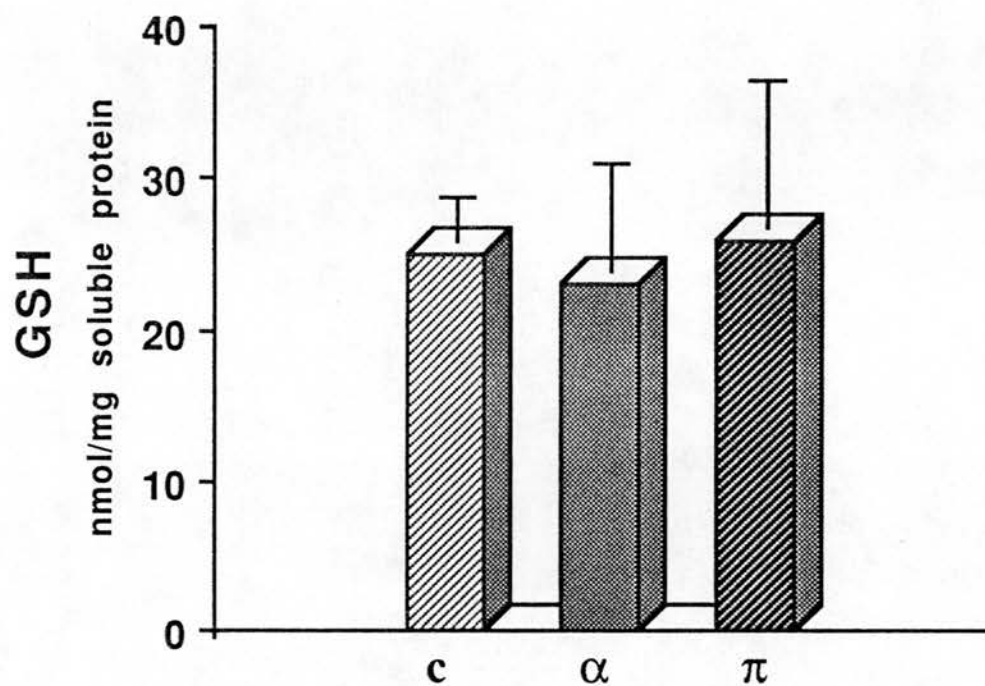


Fig.4.10. Glutathione (GSH) Levels in S.cerevisiae.

The GSH level of the three yeast strains 56/Ac-3 (c); 56/B₁-6 (α); 56/Ac-4 (π) were measured according to the method of Hissin & Hilf (1976) (section 2.17.7). The mean values± standard deviation were 24.9± 2.9 (c); 23.0± 6.9 (α); 25.0± 9.7 (π) as calculated from six determinations.

late 1960s (Young et al, 1981). These compounds now play a major role in the effective treatment of acute leukaemia, non-Hodgkin lymphomas, breast cancer, Hodgkins disease and sarcomas (Young et al, 1981). Adriamycin itself is a glycoside antibiotic originally isolated from the fungus Streptomyces peucetius. The molecule contains an amino sugar, daunosamine, linked through a glycoside bond to adriamycinone, a red-pigmented naphthacenequinone nucleus. The use of adriamycin in cancer treatment is hampered by the conventional toxicities associated with anticancer drugs, haematopoietic suppression, nausea, vomiting as well as a unique cardiomyopathy toxicity. This effect on cardiac tissue is thought to be caused either by binding of adriamycin to the cell membrane of the cardiac cell or by redox cycling and generation of toxic free radicals (see later).

The anthracyclines produce a wide range of biochemical effects that have potentially toxic consequences. This complexity has made it difficult to assign a given biochemical action to specific host tissue toxicity or tumour cell killing. At present there are three mechanisms of action ascribed to adriamycin (Young et al, 1981):-

A. DNA Intercalation

Adriamycin binds tightly (intercalates) to DNA which has several consequences including the blockage of DNA, RNA and protein synthesis as well as the inhibition of DNA repair (Painter, 1978). Some of these effects are due to adriamycin-induced DNA fragmentation, e.g. double and single-strand breaks. This strand breakage has been shown to be linked to some degree with the interaction of adriamycin with DNA topoisomerase II (Tewey et al, 1984). It is thought that this interaction has two effects, it prevents the DNA unwinding activity of DNA topoisomerase II and it stabilises the cleavable complex which leads to DNA double-strand breaks. Adriamycin can also cleave DNA via a free radical mechanism (Tewey et al, 1984).

B. Membrane Binding

It has been shown (Tritton & Lee, 1982) that adriamycin can damage cell membranes. This observation is based on the finding that adriamycin linked to large polymeric agarose beads, which prevents drug uptake, is still cytotoxic.

C. Free Radical Formation

Many studies (Bachur et al, 1982; Meijer et al, 1987; Cervantes et al, 1988) have evaluated the ability of adriamycin to catalyse the formation of oxygen radicals.

Bachur et al (1982) have shown that the nuclei of rat liver cells can catalyse a single electron reduction process of adriamycin to give free radicals. The enzymes involved being NADPH-dependent cytochrome P-450 reductase and xanthine oxidase. These free radicals react with cellular DNA and as a consequence are cytotoxic. The adriamycin semiquinone free radical produced by this bioactivation can, in the presence of molecular oxygen, produce superoxide anions, hydrogen peroxide, and hydroxyl radicals. These latter highly reactive products are the ultimate toxic species which in addition to reactions with DNA, can attack membrane lipids to produce lipid hydroperoxides which are mutagenic. Cervantes et al, (1988), using an ovarian cell line sensitive, A2780, and resistant A2780^{AD}, to adriamycin have shown that only hydroxyl radicals and not superoxide anion or hydrogen peroxide play a role in the antitumour effects of this compound. In the studies here adriamycin was initially chosen as a test compound because it has been reported that an MCF-7 cell line made resistant to this drug over-expressed GST pi (Batist et al, 1986; Cowan et al, 1986). As other biochemical changes were also observed in this cell line, it was important to establish whether the over-expression of GST pi is directly involved in the resistance mechanism or merely a casual phenomenon. It was also

possible that other GST isoenzymes play a role in protection against adriamycin particularly in view of their function as peroxidases.

To answer these questions adriamycin toxicity was determined in the control (56/Ac-3), GST pi (56/Ac-4) and GST alpha (56/B₁-6) yeast strains. Concentrations of up to 80 µg/ml adriamycin were used as described in Section 2.21. A typical survival curve obtained is shown in Figure 4.11. The concentration of drug which kills 50% of cells (LD₅₀) were 5µg/ml, 15 µg/ml and 40 µg/ml for the control, GST pi and GST B₁B₁ expressing cells respectively. On this basis the GST pi and alpha strains are 3-and 8-fold more resistant than control. The differences in sensitivity between the three strains were found to be most pronounced at the higher drug concentrations. A dose of 80 µg/ml killed 96.4% of the control cells but approximately 40% of the cells expressing GST B₁B₁ survived, 15% of the cells expressing GST pi also survived this dose. The increase in resistance in the GST alpha and pi class GST expressing strains at this dose were 11.1- and 4.2-fold respectively. This finding is important since high levels of cell kill are necessary in order to observe marked therapeutic benefits in patients receiving chemotherapy. In view of the narrow therapeutic index of anticancer drugs, because of cytotoxicity to normal

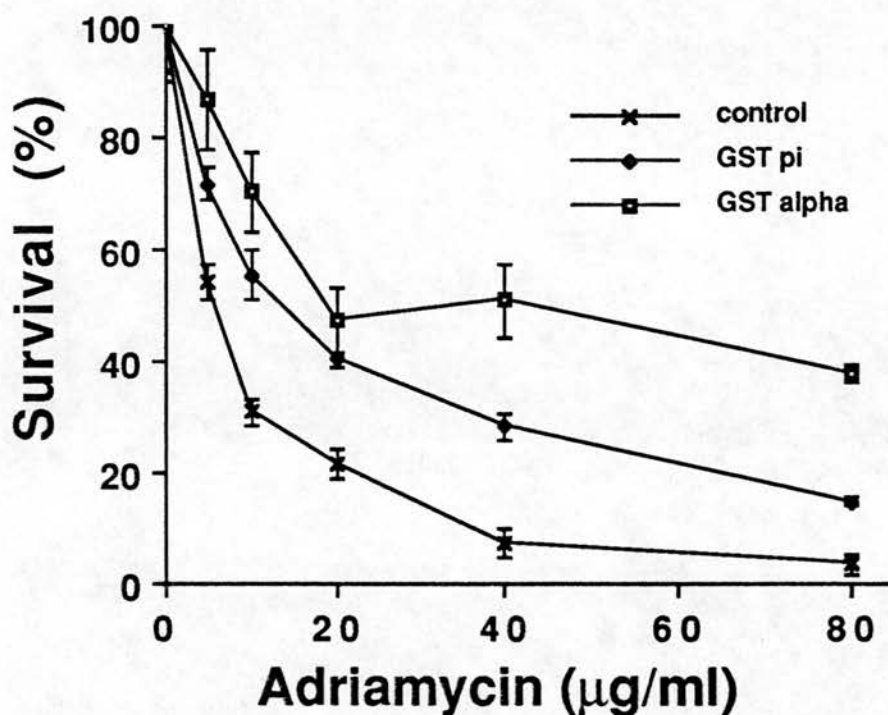


Fig.4.11. Sensitivity of *S.cerevisiae* Strains to Adriamycin.

The three yeast strains (control; GST alpha ;GST pi) were exposed to concentrations of adriamycin up to 80μg/ml as detailed in section 2.21. The activity of each strain was determined as 9.7 units (control); 103.3 units (GST alpha); 597.3 units (GST pi) using CDNB as substrate. The data are expressed as a percentage \pm standard deviation of the survival obtained in cultures where no adriamycin was added. The survival curve obtained is representative of the results obtained in three other experiments.

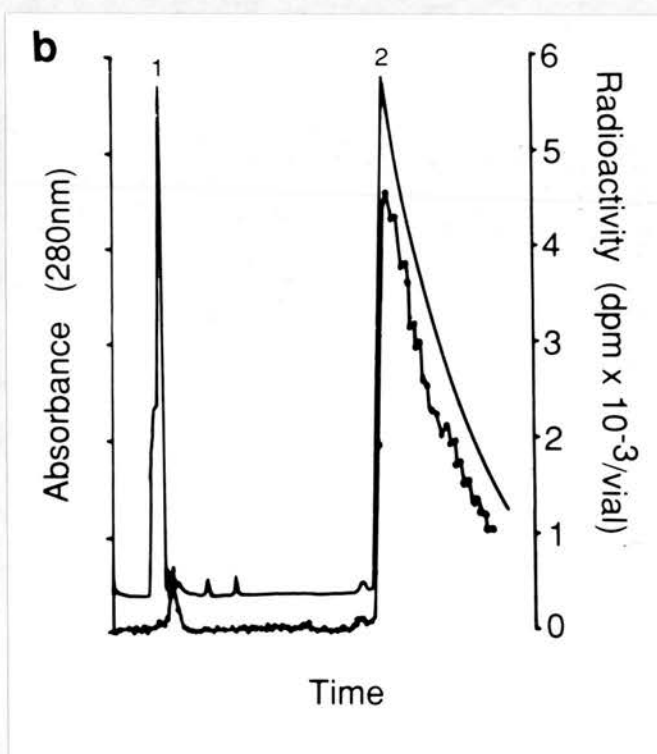
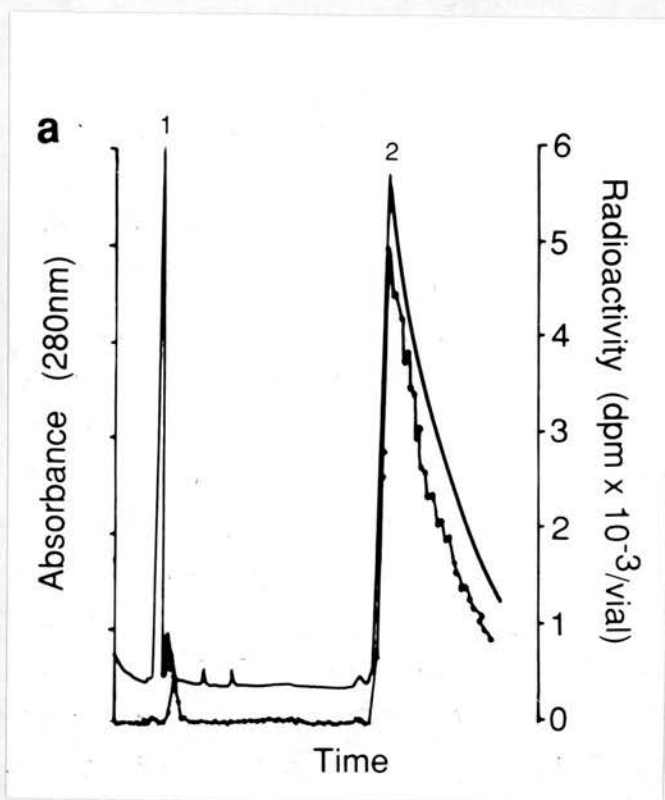
cells, even small (2-fold) changes in drug sensitivity may make a patient completely refractory to further treatment, i.e. drug resistant.

The shape of the dose-response curve obtained with adriamycin was biphasic with an initial rapid decrease in cell viability followed by a more gradual decline. This is not unexpected when the multiple cytotoxic effects of adriamycin are considered (DNA intercalation, membrane binding, free radical formation). The above data demonstrate that both GST pi and GST B₁B₁ can play a role in the development of resistance to adriamycin. This cannot be due to GST-induced changes in cellular GSH levels (4.12). Neither GST B₁B₁ nor GST pi have been shown to catalyse conjugation of adriamycin to glutathione (Ketterer, 1988). The method of resistance may not be due to a classical detoxication reaction involving GSH conjugation. GST have been shown to act as a drug or carcinogen binding proteins (Ketterer *et al*, 1967) and such binding and sequestration of adriamycin may represent the mechanism of resistance. To test this ¹⁴C-labelled adriamycin was mixed with pure mouse GST pi or GST B₁B₁ and the mixture subjected to gel permeation chromatography (Figure 4.12).

However, no radioactivity was found to be associated with the GST peaks. Thus neither GST pi nor GST B₁B₁ has the capacity to bind adriamycin. Alternatively GST

Fig.4.12 GST-Binding Assay using Adriamycin as Substrate.

In order to establish whether the alpha or pi class GST could bind adriamycin, GST B₁B₁(150μg)(a) or mouse GST π (100μg)(b) were incubated in 200μl of PBS overnight at 4°C with ¹⁴C-labelled adriamycin (100nmol, 125000dpm) (Amersham). The mixture was applied to a protein PAK Gloss SW (Waters) gel filtration HPLC column. Samples were eluted using 20mM sodium phosphate buffer, pH6.5. The GST and adriamycin peaks were monitored at 280nm. Fractions (1ml) of the eluate were collected in scintillation vials and the radioactivity content determined by liquid scintillation counting. Peak 1 represents the elution of the GST proteins from the column and peak 2 that of the radiolabelled adriamycin. The small peak of radioactivity seen just after the GST elution peak is seen when adriamycin is added to the column in the absence of protein. Thus it does not represent binding of adriamycin to the GST protein. This work was carried out by Dr. A. Bartoszek (ICRF Laboratory of Molecular Pharmacology and Drug Metabolism) and Dr J.D. Hayes (Dept. Clinical Chemistry, University of Edinburgh).



mediated resistance may be via cellular protection against adriamycin-induced peroxidative attack.

Many of the GST-mediated protection mechanisms involve glutathione and to further delineate the mechanism of resistance the cellular GSH levels were depleted using buthionine sulfoximine (BSO). BSO is an inhibitor of γ -cysteinyl synthetase a key enzyme in GSH synthesis. Cultures were treated overnight with BSO and cytotoxicity assays carried out using adriamycin. The fluorometric assay of Hissin & Hilf (1976) was used to determine the level of GSH depletion in each case. The presence of BSO reduced the levels of GSH by 56%; 60 and 50% for control, GST B₁B₁ and GST pi expressing yeast cells respectively (Figure 4.13). GSH depletion had unexpected effects on adriamycin toxicity. This treatment actually reduced rather than enhanced the toxicity of this compound. This treatment did not appear to affect the resistance observed in cultures expressing GST pi but did reverse the resistance mediated by GST B₁B₁. Thus GSH appears to be only partially involved in GST-mediated protection against adriamycin. The above data suggests that mechanisms other than those involving GSH may be involved in the protection mechanism.

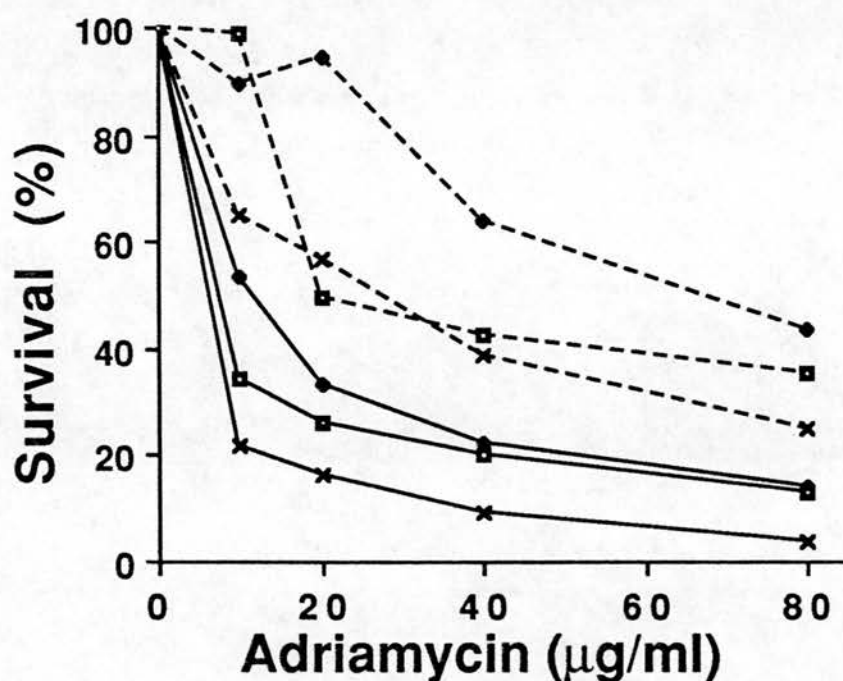


Fig.4.13. Effect of Buthionine-S,R-Sulphoximine (BSO) Treatment on Adriamycin Cytotoxicity.

The yeast strains were treated as detailed in section 2.22 using 10mM BSO. Cells were exposed to concentrations of adriamycin up to 80μg/ml (section 2.21). These cells were then compared to cultures which had not been exposed to BSO. In each case the cellular GSH concentration was determined (section 2.17.7). BSO treatment reduced the level of GSH by 56, 60 and 50% for control, GST alpha and GST pi respectively (relative to untreated cultures). Dashed lines indicate cultures exposed to BSO and solid lines those untreated with BSO. -- x -- = control ; —□— and —◇— represent cultures expressing GST alpha (B₁B₁) and GST pi respectively. This experiment was carried out in triplicate with less than 10% variation between values and repeated twice with the same effects observed.

4.14.2 Effect of GST Expression on Chlorambucil Toxicity

Nitrogen mustards, which include the widely used anticancer drug chlorambucil, are amongst the oldest antineoplastic agents in clinical use. It is believed that these compounds like other alkylating anticancer drugs, act by reacting with DNA to produce either monoalkylation products or highly cytotoxic cross-links (Calabresi & Parks, 1980). Chlorambucil reacts spontaneously to form an electrophilic carbonium ion which will react with nucleophilic centres such as DNA or protein in the absence of enzymes (Hemminki & Kallama, 1986). This compound does not, therefore, require metabolic activation to exert its toxic effects unlike cyclophosphamide. Thus the chlorambucil-induced cross-links in DNA can be either inter- or intra-strand, and can also involve DNA-protein cross-links. It is thought that the cytotoxicity of alkylating agents is directly related with their cross-linking efficiency (Roberts, 1978). In support of this conclusion bifunctional alkylating agents, such as chlorambucil, are more cytotoxic than their monofunctional counterparts (McCann *et al*, 1971; Connors, 1975). Hemminki & Kallama (1986) in an *in vitro* study with three nitrogen mustards of clinical relevance (cyclophosphamide, melphalan and chlorambucil) have shown that the major DNA cross-links formed by the action of these compounds is through the N-

7 position of guanine. Chlorambucil was chosen in this study primarily because cell lines made resistant to this compound have been shown to over-express alpha class GST isozymes (Robson et al, 1987; Buller et al, 1986). Robson et al (1987) showed that the total level of GST in the chlorambucil-resistant line (CHO-Chl^r) was elevated 3-fold (as determined with CDNB) with activity against ethacrynic acid (specific for pi class GST) and cumene hydroperoxide (specific for alpha class GST) raised 2- and 5-fold respectively. The alpha class Yc subunit has also been shown to be elevated in a Walker 256 rat breast carcinoma cell line resistant to chlorambucil (Buller et al, 1986; Clapper et al, 1987). Many other changes have also been measured in the Walker cell-line including increased DNA repair capacity thus the exposure of the yeast strains to chlorambucil should answer whether the alpha and/or pi class human GST are directly involved in resistance to chlorambucil. A typical cytotoxicity curve obtained on exposure of the yeast strains to chlorambucil is shown in Figure 4.14. Calculation of the LD₅₀ values for each strain were 100; 200 and 450 µg/ml for control, GST pi and GST B₁B₁ expressing strains respectively. This corresponds to a 2-fold and 4.5-fold increase in resistance to chlorambucil.

As with adriamycin, it is at the higher levels of drug that the differences in sensitivity between the

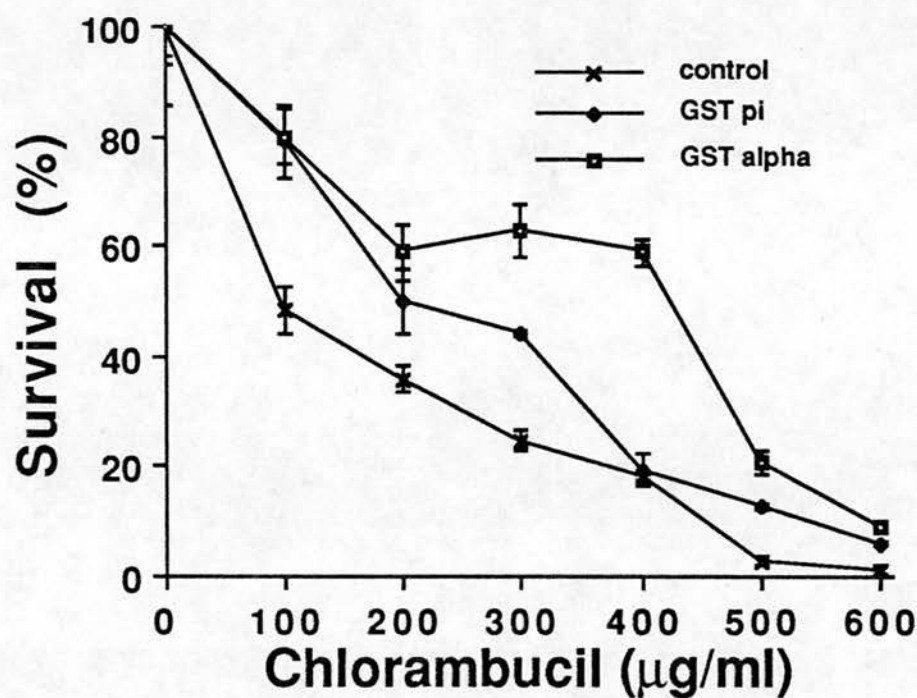


Fig.4.14. Sensitivity of *S.cerevisiae* Strains to Chlorambucil.

The three yeast strains were exposed to concentrations of chlorambucil up to 600μg/ml as detailed in section 2.21. In this experiment the activities of each strain (using CDNB as substrate) were determined as 5.2 units (control); 137.7 units (GST alpha); 407.9 units (GST pi). The data are expressed as a percentage \pm standard deviation of the survival obtained in cultures where no chlorambucil was added. The curve shown is representative of the results obtained in five other experiments.

control and GST producing strains were most marked. At a concentration of 500µg/ml 97% of the control cells were killed. However, 15% of the cells containing the pi class enzyme and 27% of the GST B₁B₁ expressing strain survived, i.e. a 5-fold and 9-fold reduction in sensitivity in relation to the control.

Although chlorambucil has not been shown to be a substrate for the alpha or pi class GST it is known that chlorambucil will react with GSH (Wolf et al, 1987). Since it is expected that GST will increase the rate of this reaction it is feasible that chlorambucil is a substrate for this enzyme system. Further evidence for this comes from the work of Dulik et al (1986) who have shown that melphalan (an aromatic nitrogen mustard like chlorambucil) will form GSH-conjugates in the presence of GST (both cytosolic and microsomal forms). To determine if GSH was involved in the observed resistance in the yeast cells BSO was again used to deplete the cellular levels of this cofactor. This treatment reduced content by 82.9, 66.5 and 73.3% in control, GST pi and GST alpha (B₁B₁) expressing cells respectively (Figure 4.15). In cultures which had not been exposed to BSO the GST B₁B₁ and GST pi expressing strains were significantly more resistant than the control strain. BSO-treatment significantly sensitised all the cultures to the toxic effects of chlorambucil. In addition exposure to BSO

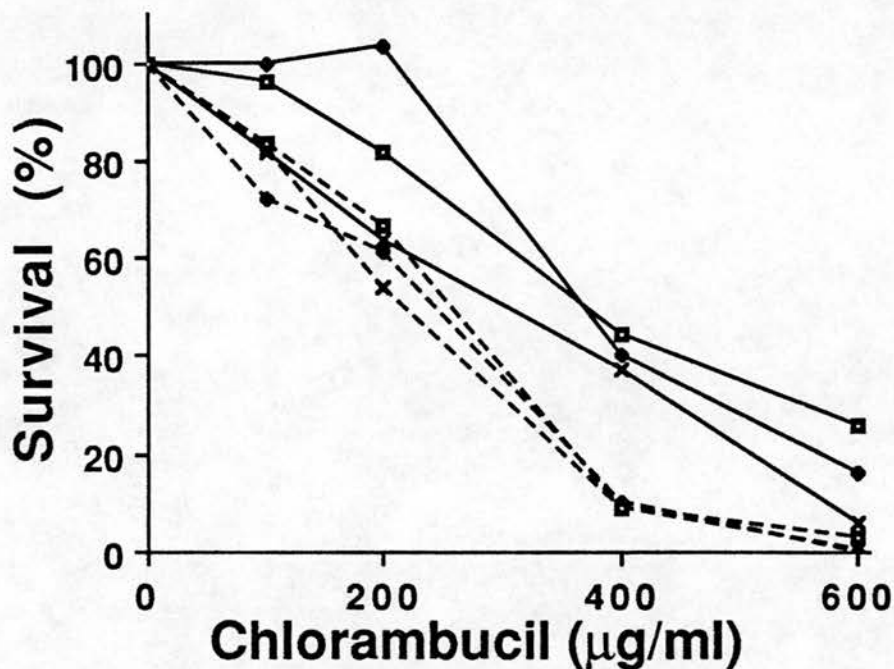


Fig.4.15 Effect of Buthionine-S,R-Sulphoximine (BSO) Treatment on Chlorambucil Cytotoxicity.

Cell cultures were treated as detailed in section 2.22 using 10mM BSO. Cells were then exposed to chlorambucil (section 2.21) at concentrations up to 600μg/ml. These were then compared to cultures which had not been exposed to BSO. In each case the cellular GSH concentration was determined (section 2.17.7). BSO treatment reduced the GSH level by 83,73 and 67% for control, GST alpha and GST pi respectively (relative to untreated cultures). Dashed lines indicate cultures exposed to BSO. Solid lines those untreated with BSO. ---x--- = control ; —■— and —◆— represent cultures expressing GST alpha (B₁B₁) and GST pi respectively. This experiment was carried out in triplicate with less than 10% variation between values and repeated twice with the same effects observed.

completely abolished the difference in sensitivity between the three strains. The fact that resistance to chlorambucil can be reversed by depletion of cellular GSH indicates that glutathione is important in the mechanism of toxicity of this compound. This result does not provide direct evidence that GST-mediated conjugation is involved in the resistance observed. Although coupled to the fact that there is no significant difference in GSH levels between the three strains is strongly suggestive that this is, in fact, the case.

4.14.3 Effect of GST Expression On 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) Toxicity

BCNU is the preferred chemotherapeutic agent for human brain tumours as it is able to cross the blood-brain barrier (Evans et al, 1987). However, more than 60% of brain tumours show insensitivity to BCNU and others acquire resistance during therapy. One major resistance mechanism is the cellular capacity to repair O⁶-alkylguanine DNA adducts (Bodell et al, 1984) but other mechanisms are thought to exist. This may include an increase in drug inactivation. BCNU has been shown to be inactivated by a denitrosation reaction that is catalysed by both the cytochrome P-450 monooxygenase system (Hill et al, 1975) and the GST (Talcott & Levin, 1983). So are the GST pi and GST B₁B₁ involved in

resistance to BCNU? The result of a cytotoxicity assay using BCNU is shown in Figure 4.16. Neither human GST pi nor GST B₁B₁ expression appears to confer resistance to this compound. This is an agreement with recently published work of Smith et al (1989) who have shown that only the rat mu class isoenzymes (3-3 and 4-4) have significant activity towards BCNU as substrate while the alpha class enzymes (1-1 and 2-2) and pi class (7-7) had only weak activity.

4.14.4 Effect of GST Expression on 1-Chloro-2,4-

Dinitrobenzene (CDNB) Toxicity

CDNB is used as a general GST substrate. CDNB is also mutagenic in the gram negative bacterium, S.typhimurium (Summer & Goggelmann, 1980) where a rapid reaction is observed between GSH and CDNB. This was initially thought to be a deactivation reaction for this compound. More recent studies using a TA100 strain of S.typhimurium and a derivative that had only 10% wildtype levels of GSH (NG-57) has shown that CDNB is actually more mutagenic in the GSH⁺ strain (TA100) (Kerklaan et al, 1985; Kerklaan et al, 1987). Further work from the same group has shown that CDNB cytotoxicity was increased in a rat liver cell line (RL-4) when intracellular GSH levels were increased (two-fold) by treatment with the monoethyl ester of glutathione (EGSH). There was also a

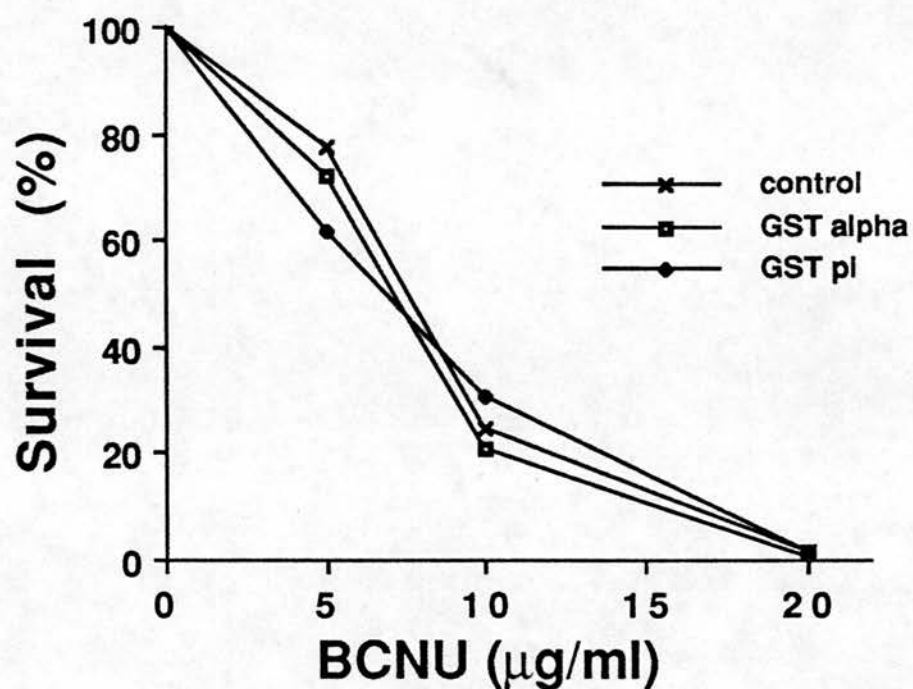


Fig.4.16 Sensitivity of *S.cerevisiae* Strains to 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU).

The three yeast strains were exposed to concentrations of BCNU up to 20μg/ml as detailed in section 2.21. The activities of each strain (using CDNB as substrate) were determined as 8.5 units (control); 130.2 units (GST alpha); 420.6 units (GST pi). This experiment was carried out in triplicate with less than 10% variation between values.

corresponding decrease in CDNB induced cell-death when diethylmaleate (DEM) was used to decrease GSH (to 20% of control levels) (Bruggeman et al, 1988). There is, therefore, contradictory information on the role of GSH in protecting against CDNB. A role for the GST in the protection against CDNB toxicity has not been reported, however it has been shown that an NCIH322 cell line made resistant to CDNB (Wareing et al, 1988) expresses alpha and pi class GST subunits. In order to evaluate a direct role for the GST in the resistance mechanism the human GST expressing yeast strains were exposed to CDNB. Different effects caused by CDNB were observed between the GST B₁B₁ and GST pi expressing strains (Figure 4.17). The GST B₁B₁ expressing strain is more resistant and the GST pi strain less resistant than the control. The LD₅₀ values for the three strains are 30, 55 and 70 µg/ml for the GST pi, control and GST alpha strains respectively. Similar to the findings with the anticancer drugs the differences in sensitivity were most marked at the highest drug concentrations such that at 80µg/ml CDNB the survival of the three strains were reduced to 3.2, 15.1 and 34% for the GST pi, control, and GST alpha respectively. Thus the GST pi strain is approximately 5-fold more sensitive than the control and over 10-fold more sensitive than the GST B₁B₁ expressing strain. The GST alpha strain is seen to be 2.3-fold more resistant to

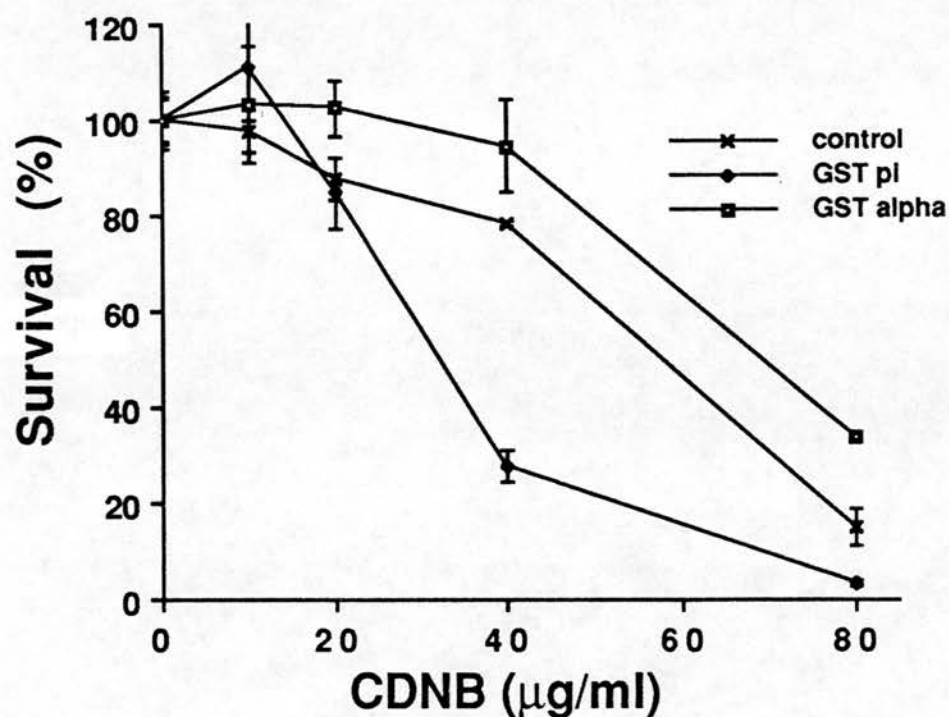


Fig.4.17 Sensitivity of *S.cerevisiae* Strains to 1-Chloro-2,4-Dinitrobenzene (CDNB).

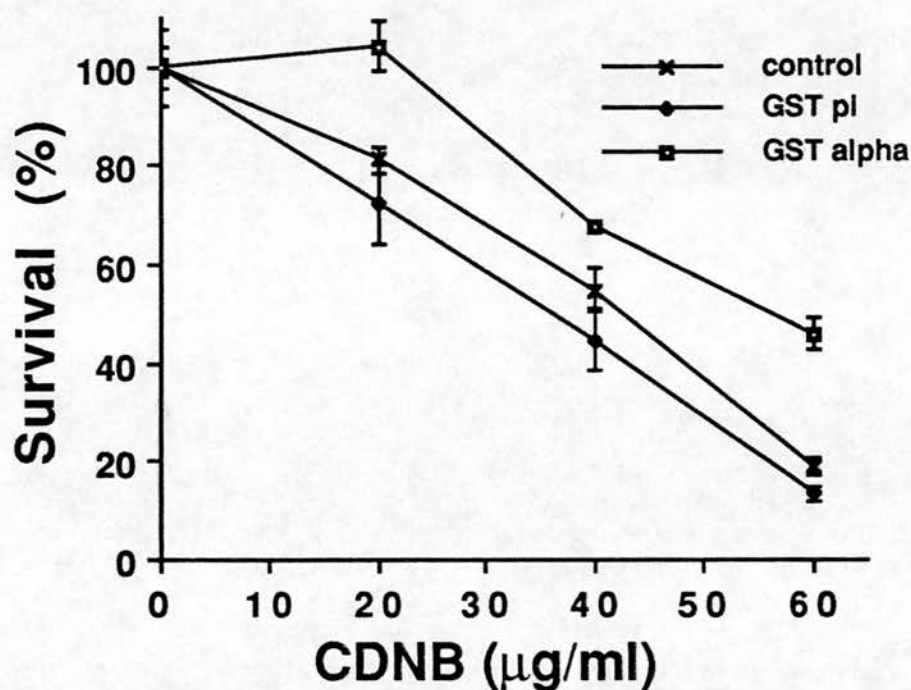
The three yeast strains were exposed to concentrations of CDNB up to 80μg/ml as detailed in section 2.21. The activity of each strain was determined (using CDNB as substrate) as 5.2 units (control); 137.7 units (GST alpha); 407.9 units (GST pi). The data are expressed as a percentage \pm standard deviation of the survival obtained in cultures where no CDNB was added. The curve shown is representative of the results obtained in three experiments.

the cytotoxic effects of CDNB than the control strain.

The difference in sensitivity between the B₁B₁ expressing strain and the control strain is much less than for either chlorambucil or adriamycin. The reasons for this is unknown. The mechanism(s) of cellular toxicity induced by CDNB are unknown and likely to be complex. It is known for example that FDNB, the fluoro-derivative of CDNB, can inactivate the transport of glucose into the cell resulting in cell death (Walmsley, 1988). This suggests that some of the cytotoxic effects of CDNB may occur outside the cell and outwith the range of detoxification (or toxification) by GST. This may lead to the observed smaller differences in sensitivity between the yeast strains. The two GST expressing strains also have different effects on the sensitivity of the host cell to CDNB exposure. Over-expression of the human alpha class GST confers resistance while the pi class GST does not. Both human enzymes have high activity towards CDNB as substrate with alpha having $64\mu\text{mol min}^{-1}\text{mg}^{-1}$ and pi, $105\mu\text{mol min}^{-1}\text{mg}^{-1}$ (for the purified enzymes) (Mannervik, 1985). Thus if GST conjugation to CDNB was a toxification reaction then both strains would be expected to be more sensitive than the control which has very low endogenous activity towards CDNB. One possible explanation is that the alpha class enzyme induced resistance is not due to conjugation

to GSH but to some other mechanism. This mechanism may be the covalent binding of CDNB to the protein. This would be a detoxification reaction as it would reduce the active drug concentration. This has been reported to occur at a very high rate with purified rat liver GST isoenzymes where up to 30% of the GST can be found covalently bound to CDNB after a 5 min incubation (Jakoby, 1978). In order to evaluate the role of GSH in CDNB toxicity BSO was used to deplete cellular GSH. If the mechanism of resistance seen in the alpha expressing strain is due to covalent binding of CDNB to the GST protein then the level of resistance would be independent of cellular GSH. In the case of the pi class GST if the conjugation of GSH to CDNB is the mechanism of sensitisation then depletion of GSH should reverse this effect. This result is shown in Figure 4.18.

The GST B₁B₁ expressing strain was still approximately 2-fold more resistant than control after BSO treatment. However, the depletion of GSH altered the sensitivity of the GST pi expressing strain such that it is no longer more sensitive than control CDNB exposure. Thus GSH appears to play a role in GST pi-induced sensitivity to CDNB while the GST B₁B₁ conferred resistance is GSH independent. This result is consistent with the alpha class GST enzyme protecting the cell by covalent binding to the CDNB molecule.



**Fig.4.18 Effect of Buthionine-S,R-Sulphoximine (BSO)
Treatment on CDNB Cytotoxicity.**

The yeast strains were treated as detailed in section 2.22 using 10mM BSO. Cells were exposed to concentrations of CDNB up to 80 $\mu\text{g/ml}$ (section 2.21). The cellular GSH concentrations were determined and the level of GSH depletion calculated by comparison to untreated cultures. GSH levels were reduced by 70, 72 and 60% for control, GST alpha and GST pi respectively. The results are expressed as percentage survival \pm standard deviation of the survival obtained in cultures where no CDNB was added. This experiment was repeated twice with the same effects observed.

4.14.5 Effect of GST B₁B₁ Expression on Cumene Hydroperoxide (CHP) Toxicity

Previous work has shown that the alpha class of GST have high selenium-independent peroxidase activity towards the organic hydroperoxide, cumene hydroperoxide (CHP) (Lawrence & Burk, 1978; Lawrence *et al*, 1978; Prohaska, 1980). This activity has been shown to be distinct from the selenoenzyme (selenium-dependent) glutathione peroxidase (Lawrence *et al*, 1976).

This work has been advanced by Wareing *et al* (1988) where an NCIH322 cell-line resistant to CDNB is also cross-resistant to CHP. For this reason the 56/B₁-6 strain expressing the human B₁B₁ protein was exposed to CHP and the sensitivity compared to the control strain (56/Ac-3). The human pi class expressing strain (56/Ac-4) was not tested as this enzyme has very low peroxidase activity towards CHP (Section 4.12). The result shown in Figure 4.19 indicates that the human alpha class enzyme (B₁B₁) can protect cells against the cytotoxic effects of organic hydroperoxide exposure. The increased level of resistance compared to the control is seen to be around 2-fold at 100µM CHP. This is in agreement with the work of Wareing *et al* (1988) where a 2.5-fold increase in the LD₅₀ levels of a CDNB-resistant NCIH322 cell-line has been observed on exposure to CHP.

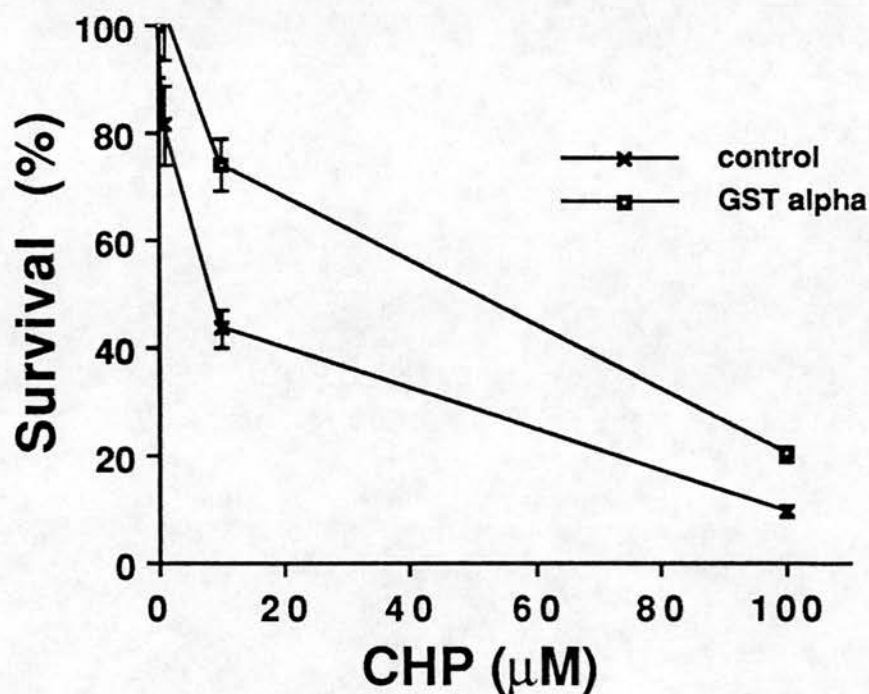


Fig.4.19 Sensitivity of *S.cerevisiae* Strains to Cumene Hydroperoxide (CHP).

The yeast strains were exposed to concentrations of CHP up to 100 μM as detailed in section 2.21. The activity towards CDNB as substrate was determined as 9.7 units for control and 89.6 units for GST alpha. In this experiment the GST pi expressing strain (56/Ac-4) was not tested as substrate. The results are expressed as percentage survival \pm standard deviation of the survival in cultures where no CHP was added. The curve shown is representative of those obtained in the three times this experiment was carried out.

4.14.6 Effect of GST Expression on 1,2-Dibromoethane (DBE) Toxicity

Conjugation to glutathione is obviously an important detoxification reaction for electrophilic xenobiotics. But despite its primary detoxifying role GSH-conjugation to certain compounds results in their transformation into more reactive and hence toxic derivatives. These can be grouped into three such that the conjugate:-

- 1) is itself the reactive species
- 2) is further metabolised to the reactive species
- 3) releases reactive species where the conjugation reaction is reversible.

There are examples of all three types.

The vicinal dihalogenoalkane 1,2-dibromoethane (DBE) belongs to the first type such that the S-2-bromoethylglutathione species formed is directly active. DBE was first produced in 1826 and is now widely used as a soil fumigant as well as a lead scavenger in petrol containing tetra-alkyllead and antiknock preparations. DBE has been shown to cause base pair substitutions in S.typhimurium even in the absence of an exogenous metabolising system (Rannug, 1980). However, the mutagenicity is considerably increased by the addition of rat liver cytosol to the incubation mixture (van Bladeren, 1988). It has been shown that the GSH-DBE

conjugate causes in vitro unscheduled DNA synthesis (UDS) in F-344 rat pachytene spermatocytes and hepatocytes as well as in vivo UDS when given i.p. to rats (Working et al, 1986). If GSH levels were depleted with DEM then DBE-induced UDS was not observed in vitro (Working et al, (1986).

DBE was thus chosen to see if the expression of human GST's within the yeast cell, would cause an increase in DBE toxicity. The result is shown in Figure 4.20. Both the alpha and pi class GST expressing strains are more sensitive to the toxic effects of DBE than the control strain. The strain expressing GST alpha has a much steeper fall in survival than the GST pi strain suggesting that human alpha class enzymes catalyse the conjugation of GSH to DBE to a greater extent than GST pi. The GST pi and B₁B₁ expressing strains were found to be 2.5- and 3-fold more sensitive than control to the toxic effects of 800µg/ml DBE.

This result demonstrates that in certain circumstances (analogous to some cytochrome P-450 oxidation reactions) the GSH conjugates formed by GST action can lead to the formation of highly toxic metabolites.

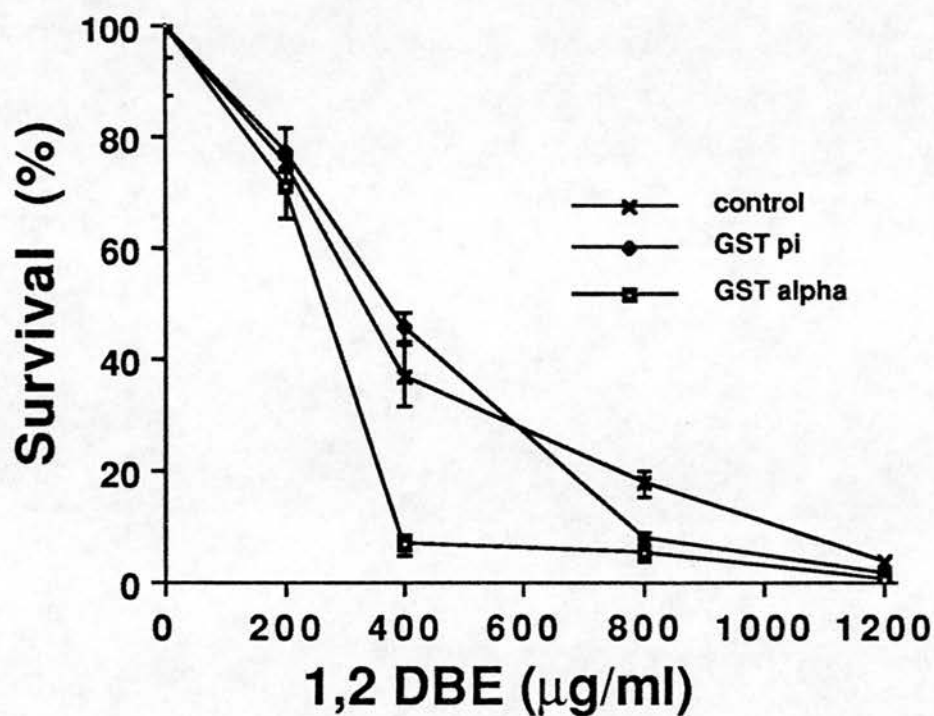


Fig.4.20 Sensitivity of *S.cerevisiae* Strains to 1,2-Dibromoethane (DBE).

The yeast strains were exposed to concentrations of DBE up to 1200μg/ml (as detailed in section 2.21). The activity of each strain was measured using CDNB as substrate and found to be 8.3 units (control), 119.5 units (GST alpha) and 394.4 units (GST pi). The results are expressed as a percentage \pm standard deviation of the survival obtained in cultures where no DBE was added. Similar results were obtained when this experiment was repeated.

4.15 Co-Expression of Human Pi and Alpha Class GST in *S.cerevisiae*

The experiments described above (4.14.1) indicated that the mechanism of action of the pi and alpha class GST towards adriamycin may be different. This raised the possibility that when expressed in the same cell they may significantly increase the resistance towards this compound (most human cells express both these proteins simultaneously). Experiments were therefore carried out to establish whether this was the case. However, it was first necessary to clone the GST pi cDNA into an expression vector with a marker other than TRP1. The isolated pi class GST cDNA was blunt-end ligated into the PvuII site of the vector pVT100-U which has the ADC1 promoter but URA-3 as the selectable marker (Vernet et al, 1987). After transformation into E.coli DH1 the colony-screening method of Grunstein & Hogness (1975) was used with ³²P-labelled GST pi cDNA as a probe to determine possible pVT100-U recombinant plasmids (Figure 4.21). Six colonies were picked at random and the plasmid DNA isolated. EcoRI restriction digests were then used to determine which plasmids contained the human pi class cDNA as insert (not shown). This gave five possible plasmids, designated pVT/Ac-1 to pVT/Ac-5 which were transformed into the S.cerevisiae strain KY118 using lithium acetate (Itoh et al, 1983). Colonies from each

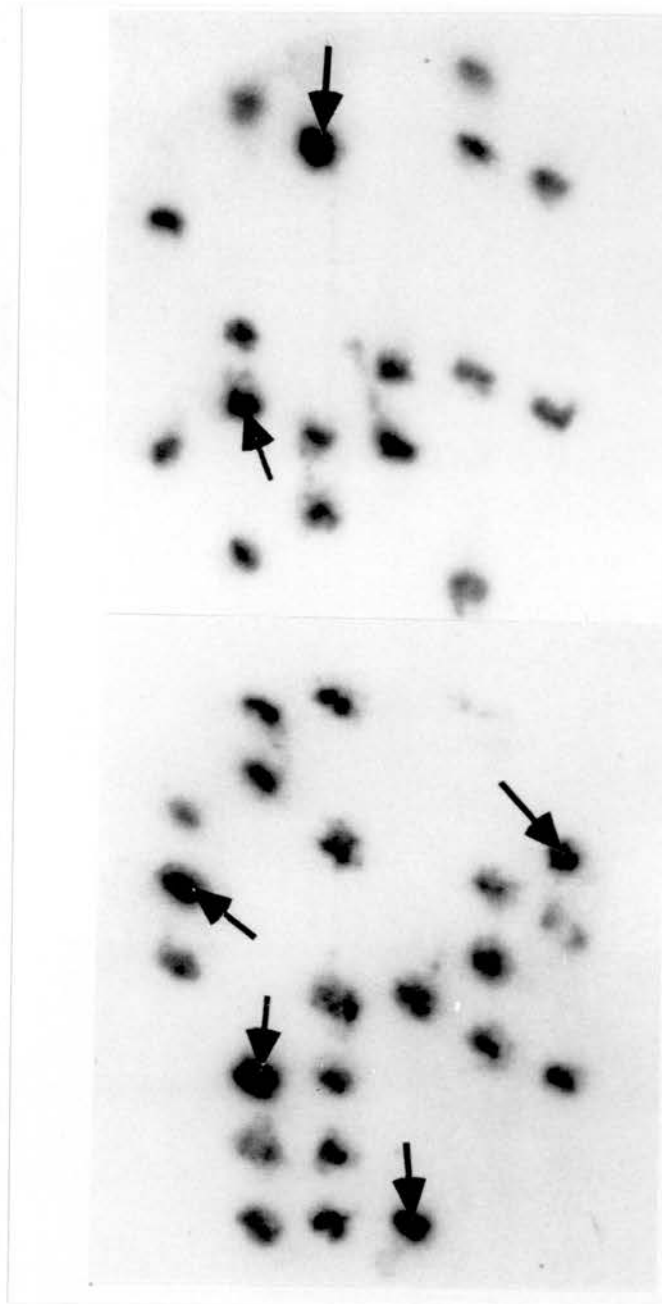


Fig.4.21 Colony Hybridisation Analysis on Transformed E.coli DH1 Cells.

The colony hybridisation method of Grunstein & Hogness (1975) (section 2.15) was used to determine which of the transformed E.coli DH1 colonies contained the pVT/Ac vector. ³²P-labelled GST pi cDNA was used as a probe and six positive colonies picked at random (marked by arrows).

transformation were grown in supplemented synthetic SD medium. Cytosol fractions were prepared and subjected to SDS-page and Western blotting with the GST antisera (Figure 4.22). Only the plasmid designated pVT/Ac-4 gave a cross-reacting band with the human GST pi antibody. This plasmid was then used to transform the p56/B₁-6 strain (expressing the human B₁B₁ protein). Western blot analysis of the resulting strain, designated B₁Ac, is shown in Figure 4.23. Two bands which co-migrated with both the purified human GST B₁B₁ and GST pi standards were observed. Thus it is possible to express both proteins from different vectors providing each has its own unique selectable marker. Simultaneous expression of two proteins in *S.cerevisiae* has also been reported by Murakami et al (1986) who simultaneously expressed cytochrome P4501A1 and P-450 reductase. However, in their system both expressed cDNAs were arrayed in tandem on the same plasmid. In the experiments here the level of GST expression from the two different vectors seemed to vary between experiments and in some of the experiments one of the proteins predominated.

The sensitivity of this strain relative to those expressing either GST B₁B₁ or GST pi was then established. The strain expressing both GST simultaneously had approximately 2-fold higher CDNB activity (Figure 4.24) (350 units for B₁/Ac vs 220 units

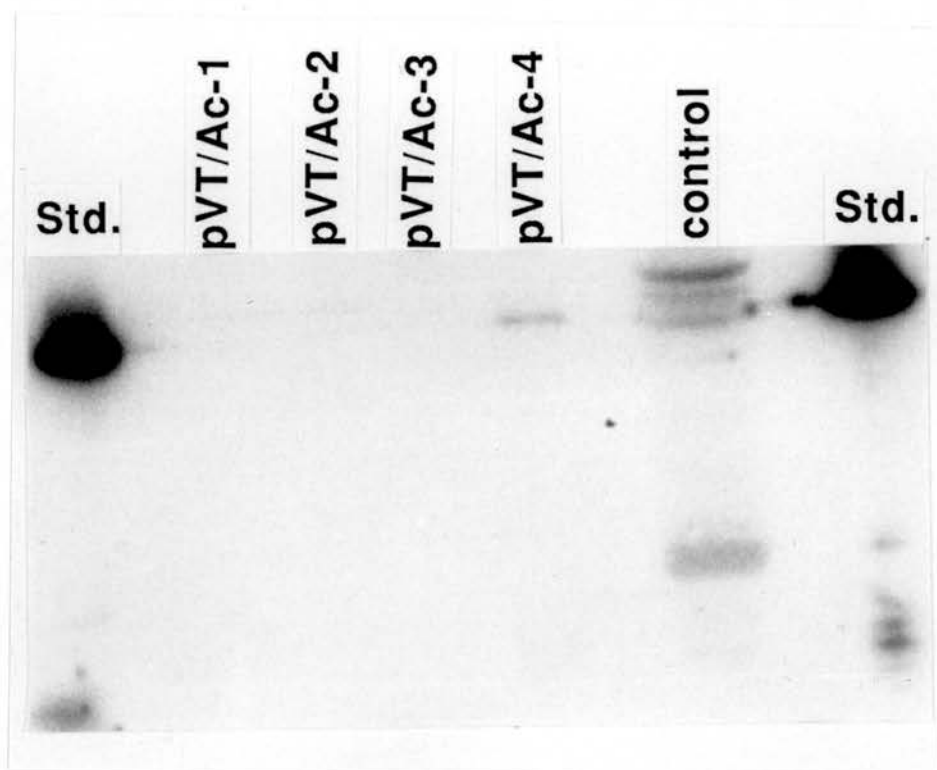


Fig. 4.22 Expression of Human Pi Class GST using pVT 100-U.

SDS-page followed by Western blot analysis was carried out on cytosolic fractions (50µg) of pVT/Ac-1 to pVT/Ac-4. An antibody raised against the human GST pi was used to identify the presence of the human GST pi protein. Purified human GST was loaded as standard. A band co-migrating with the standard is only observed in the pVT/Ac-4 transformed strain. The control is cytosol from the 56/Ac-3 transformed strain. Cytosolic fractions were prepared as in section 2.16.3.

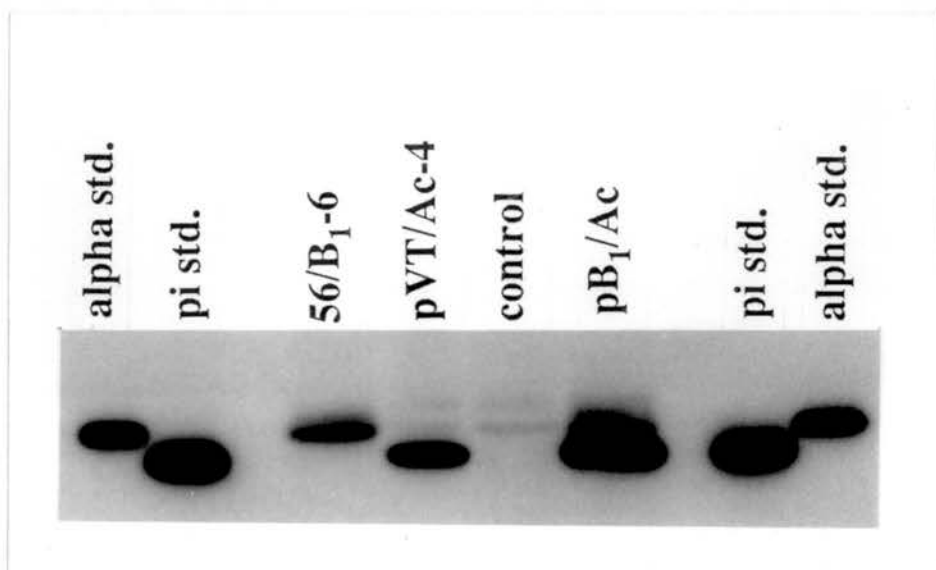


Fig.4.23 Co-Expression of Human Pi and Alpha Class GST in S.cerevisiae.

SDS-page followed by Western blot analysis was carried out on cytosolic preparations (50µg) of p56/B₁; pVT/Ac-4; p56/Ac-3 (control) and pB₁/Ac. Antibodies raised against the purified alpha (B₁B₁) and pi class GST were used to identify the proteins. The purified human proteins were loaded as standards. The pB₁/Ac strain gives two bands corresponding to the human GST pi and GST B₁B₁ proteins. The p56/B₁-6 and pVT/Ac-4 transformed strains only give single cross-reacting bands. No cross-reacting bands are seen in the control. These samples were prepared from the cultures used to give the cytotoxicity curve in Fig.4.25. Cytosolic fractions were prepared as in section 2.16.3.

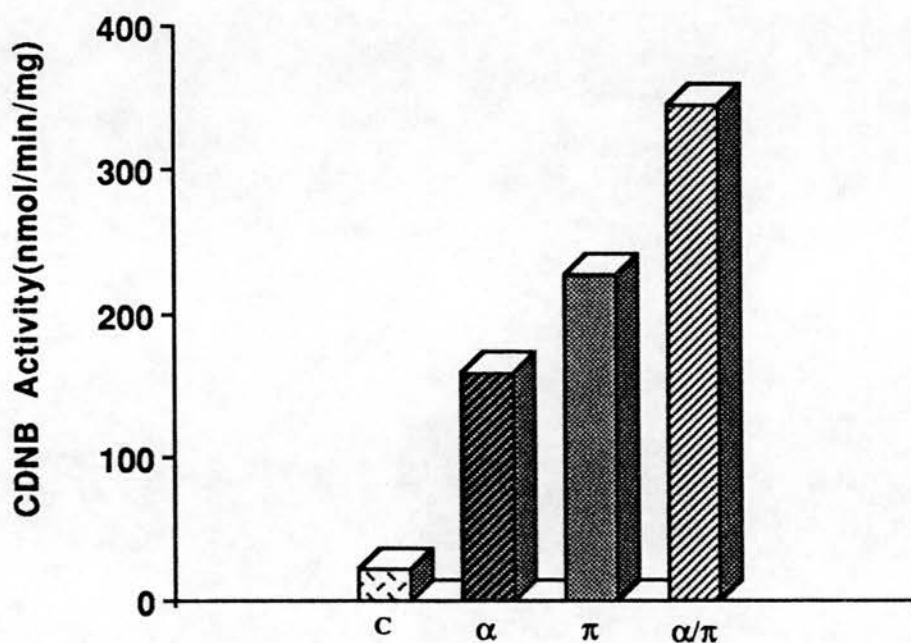


Fig.4.24 CDNB activity in Yeast Cultures Expressing Human GST.

GST activity in cultures used for the cytotoxicity assay in fig.4.25 were measured using CDNB as substrate. C = control culture, α = culture expressing GST B₁B₁, π = culture expressing GST pi and α/π = culture expressing both alpha and pi class GST simultaneously. CDNB activities are given in the text.

for the GST pi and 150 units for the GST B₁B₁ strains). The control strain (56/Ac-3) had only minimal activity towards CDNB (10 units).

Strain B₁Ac was significantly more resistant to adriamycin than either of the single GST expressing strains (2-fold) (Figure 4.25). Thus the expression of multiple GST isoenzymes within a cell appears to decrease the sensitivity of the host cell to cytotoxic insult even in comparison to cells which over-express only one GST isoenzyme. The result here suggests that this increase is additive with approximately 2-fold greater survival obtained for the double expressing strain as compared to the single GST producing strains. This may be related to the overall protein levels as the strain expressing both the human GST isoenzymes had approximately 2-fold higher activity towards CDNB (Figure 4.25).

If extrapolated the result above suggests that in a human tumour the overall levels of both alpha and pi class GST (and probably also the mu class GST) will be important in determining tumour response to the prescribed antineoplastic treatment. That the over-expression of both isoenzymes confers greater resistance to adriamycin than the expression of a single GST is interesting in that early studies indicated that pi class enzymes were predominantly over-expressed in cell-lines resistant to adriamycin (Batist et al, 1986; Cowan et al,

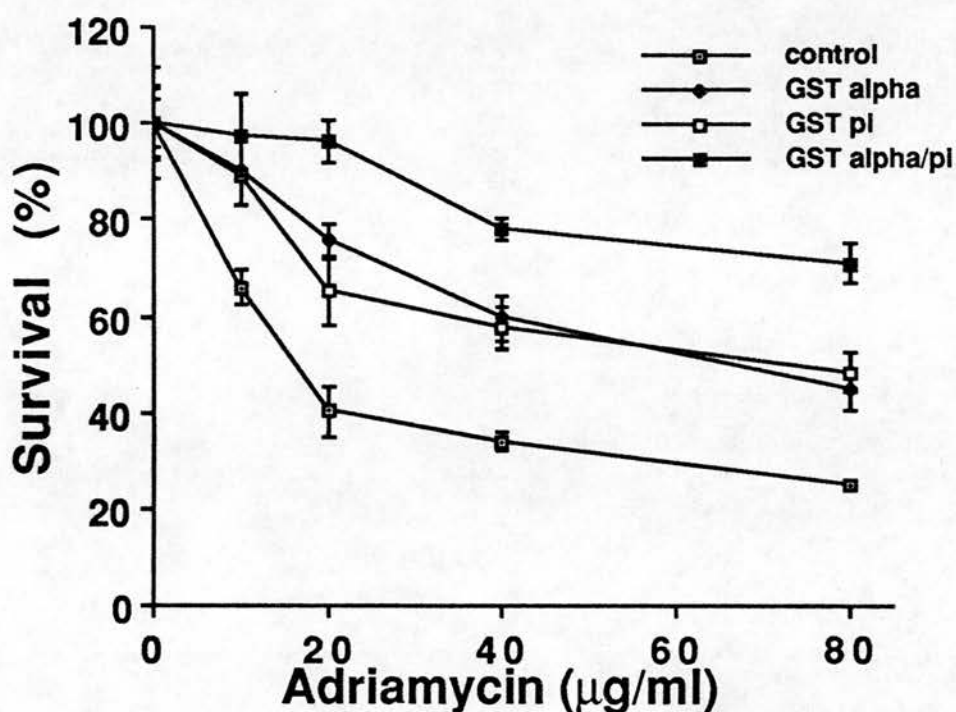


Fig.4.25 Adriamycin Cytotoxicity on *S.cerevisiae* Simultaneously Expressing Human GST B₁B₁ and GST Pi.

The yeast strains (control, GST alpha, GST pi (pVT/Ac-4) and GST alpha/pi(pB₁/Ac)) were exposed to concentrations of adriamycin up to 80 μg/ml as detailed in section 2.21. The activity of each strain is shown in Fig.4.24. The data are expressed as a percentage \pm standard deviation of the survival obtained in cultures where no adriamycin was added. The survival curves obtained were similar when the experiment was repeated.

1986). In cell lines made resistant to alkylating agents the alpha class GST was shown to predominate (Robson et al, 1987; Lewis et al, 1988b). However, more recent studies shown that both the alpha and pi class GST can be elevated in cell-lines resistant to alkylating agents (Wareing et al, in preparation). This is also true in the drug-resistant preneoplastic foci of rat livers (see Farber, 1984) where the GST of alpha, pi and mu classes are all over-expressed. Thus in a human tumour the GST enzyme which is actually over-expressed may be determined by the presence of the factors necessary for gene expression.

4.16 Discussion

There are many reports on the elevation of GST in cell lines made resistant to antineoplastic drugs (Wang & Tew, 1985; McGowan & Fox, 1986; Batist et al, 1986; Robson et al, 1987; Smith et al, 1989). However, in most cases there are a plethora of changes in enzymes other than GST. In perhaps the best study Cowan et al (1986) have shown that GST pi is over-expressed 45-fold relative to the parental cell line in an MCF-7 cell line resistant to adriamycin. However, further characterisation of the resistant cell line has shown that the phase I (toxication) cytochrome P-450 enzymes were decreased (as estimated by arylhydrocarbon hydroxylase activity).

There was also shown to be a 2-3-fold decrease in intracellular drug accumulation associated with an increase in p-glycoprotein levels. Also phase II (detoxication) enzymes other than GST were found to be over-expressed, for example UDP glucouronyl and sulpho transferases. These changes associated with adriamycin exposure thus make it difficult to assign a role for the over-expressed GST pi in the acquired resistance. This can be said of all cell line models where continuous drug exposure has been used to generate the resistant line. In an attempt to simplify this position two human GST cDNAs have been expressed in S.cerevisiae. These cDNAs encode GST B₁B₁ and GST pi. The enzymes were produced at high levels which represents a significant advantage over mammalian models where low levels of expression are obtained. The levels of GST produced in S.cerevisiae were comparable to those obtained from rat liver where up to 10% of the cytosolic protein can be GST (Jakoby, 1978). The proteins were found to be functional as determined by activity towards CDNB and CHP as substrates. Another reason for using S.cerevisiae was because yeast were reported to have no cytosolic GST activity (Jakoby, 1978) and thus the contribution of background activities would be expected to be extremely low. There is a growing body of evidence that indicates GSH levels are an important factor in determining the

susceptibility of tumour cells to a wide range of cytotoxic drugs (Calcutt & Connors, 1963; Arrick & Nathan, 1984; Hayes & Wolf, 1988). This includes alkylating agents, such as melphalan and chlorambucil, as well as quinone containing compounds, such as adriamycin and daunorubicin. GSH depletion has been shown to sensitise ovarian carcinoma cells to the toxic effects of melphalan, cis-platinum and adriamycin (Green et al, 1984; Hamilton et al, 1985). Thus the GSH levels within the strains used required to be comparable. This was indeed the case and expression of GST does not appear to influence GSH levels. The differences in cellular sensitivity to cytotoxic insult can thus be directly attributed to the action of GST within the cell.

The main object of the expression of GST in S.cerevisiae was to determine if this resulted in altered sensitivity to alkylating agents, adriamycin and hydroperoxides. The results suggest that under the correct conditions the expression of GST can confer resistance to the host cell. The alpha and pi class GST are shown here to confer up to 11-fold and 4-fold resistance to adriamycin respectively as well as 9-fold and 5-fold resistance to chlorambucil. This supports the work of Batist et al (1986) who showed the over-expression of GST pi in an MCF-7 cell line was 192-fold resistant to adriamycin. While the work of Robson et al

(1987) have shown that the over-expression of an alpha class GST in a CHO cell line confers resistance to a number of nitrogen mustards including chlorambucil. Thus the over-expression of GST appears to modulate cellular sensitivity to cytotoxic drugs. It should be noted that neither alpha nor pi class enzymes have been shown to be active towards adriamycin as substrate. However, it is suggested that both may have activity towards the lipid hydroperoxides formed by the action of free radicals produced by the quinone-semiquinone cascade induced by adriamycin metabolism (Ketterer, 1988; Tew & Clapper, 1989). Certainly the alpha class GST expressed has been shown to protect cells from the cytotoxic damage induced by organic hydroperoxides in the form of CHP (2-fold) (Wareing et al, 1988). In experiments here alpha class GST B₁B₁ expression resulted in a 2-fold resistance to CHP indicating a similarity between the cell line model and expression in S.cerevisiae (the pi class enzyme was not tested as it has no peroxidase activity towards CHP).

Only two anticancer drugs have been shown to be substrates for GST, namely melphalan and BCNU (Dulik et al, 1986; Smith et al, 1989). Thus an attempt was made to determine if GST mediated conjugation to GSH was the mechanism of resistance. To this end cells were treated with the GSH depletor BSO in an attempt to sensitise the

cells to the anticancer drugs. In the case of chlorambucil conjugation to GSH appeared to be the mechanism of detoxification. It has been proposed (Russo et al, 1986) that BSO depletion of cellular GSH could be used as a mechanism to increase tumour susceptibility to antineoplastic therapy. However, the report by Calvin et al (1986) that BSO depletion was associated with the development of cataracts in mice suggests that this is probably not feasible. However, the modulation of GSH levels by other means to increase tumour susceptibility is an avenue of research that should not be ignored.

The role of GSH in adriamycin detoxication was complicated. GSH depletion reduced GST B₁B₁ mediated resistance to adriamycin but not that mediated by GST pi. GST pi mediated protection was shown not to involve adriamycin sequestration. Thus the mechanism(s) by which the GST pi protects the yeast cells remains unclear. It is interesting to speculate on what other protection mechanisms there may be operating. The GST may be acting as stress response proteins interacting with other cellular components in a similar manner to Hsp70.

Another anticancer drug tested here was BCNU. BCNU is used as frontline therapy in the treatment of human brain tumours (Evans et al, 1987). Neither the alpha nor pi class GST protected against the cytotoxic damage induced by this compound. This is in agreement with the recently

published work of Smith et al (1989) where only the rat mu class GST (3-4 and 4-4) were shown to have dinitrosation activity towards BCNU. Neither the alpha (1-2) nor pi (7-7) enzymes exhibited significant activity. The lack of difference between yeast strains is an important result as it shows that the resistance to adriamycin and chlorambucil is a specific event. Thus the over-expression of a foreign protein does not a priori confer resistance. For example, one could imagine that the over-expression of a foreign protein could provide an increase in nucleophilic centres to which reactive electrophiles such as anticancer drugs can bind. This would result in a reduction in DNA damage, damage which is likely to be the lethal event to a cell. The fact that cells expressing human alpha or pi class GST shown no alteration in their sensitivity to BCNU suggests that the model detailed here is specific to those compounds which the GST have activity towards.

The potent alkylating agent and general GST substrate CDNB was also examined in this system. The main interest in this compound was derived from the work of Wareing et al (1988) who have shown that an NCIH322 cell line resistant to CDNB has an elevated expression of the alpha and pi class enzymes. The results here obtained support the conclusions that this expression is involved in the resistance mechanism. This small

increase in resistance is probably due to the cytotoxic damage induced by CDNB outwith the cytosol. For example, the fluoro-derivative of CDNB, has been shown to be an inhibitor of the glucose transport system with toxic consequences for the cell (Walmsley, 1988). Obviously any damage outwith the cell cannot be protected against by over-expression of a GST. This would limit the protection potential of the GST system towards this compound.

The over-expression of the pi class GST which has higher activity towards CDNB than the alpha class enzyme actually resulted in the yeast strain being more sensitive than the control. This result supports that of Van Bladder and co-workers who have shown that CDNB-GSH conjugates are actually more mutagenic in S.typhimurium than the CDNB molecule itself.

This increase in mutagenicity could be reversed if a GSH⁻ strain was used (Kerklaan et al, 1985). The over-expression of GST pi should increase the level of CDNB-GSH conjugation within the yeast which could be activated to more toxic species by the action of nitroreductases. This would result in an increase in sensitivity towards CDNB when compared to control as also seen in the cytotoxicity assay. This raises the question why does the alpha class GST protect the yeast

cell? The alpha class GST may be acting not as a GSH-conjugation enzyme but as a suicide protein forming covalent linkages to the CDNB leading to deactivating. This would produce an inactive protein but would also protect cellular targets from CDNB injury. This possibility was supported by the finding that on GSH depletion strain expressing GST pi class became as sensitive as control, possibly due to a decrease in CDNB-GSH formation, while the GST B₁B₁ expressing strain remained resistant relative to the control.

The work in this chapter has shown that although the GST family of enzymes are thought of as detoxication proteins, under certain circumstances they are shown to be capable of producing toxic species. The model compound used was DBE which after conjugation to GSH undergoes activation to form a sulphur half-mustard (Rannung, 1980; Van Bladderren, 1988). Both the human alpha and pi class GST increased the sensitivity of their host cells to DBE induced damage. Thus although in the majority of cases GST and GSH give cellular protection to electrophile-induced damage under certain conditions they can increase drug sensitivity. This fact could be exploited to produce anticancer drugs which increase tumour susceptibility over normal cells if GST levels are found to be increased.

4.17 Refinement and Potential of GST Expression in *S.cerevisiae*

One problem associated with *S.cerevisiae* as a recipient organism is that they are relatively insensitive to chemical toxins. The levels required to produce acceptable levels of cell kill are approximately 10-fold higher than that required to produce equivalent cell death in a mammalian cells. In fact, the *S.cerevisiae* strain used proved to be completely refractory to the nitrogen mustard, melphalan. Even at the limit of solubility (1600µg/ml) 90% of the control yeast cells were still viable (data not shown). An obvious way to circumvent this problem is to produce mutations affecting the cell wall in an attempt to produce a strain with a greater sensitivity to cytotoxic insult. Another method could be to pre-treat the cells with compounds which are non-lethal but increase the permeability of the yeast to other chemicals. The nonapeptide of polymyxin B sulphate reported by Boguslawski (1985) has potential in this respect. The nonapeptide is thought to puncture the yeast cell wall and membrane and facilitate the passage of agents into the cytoplasm, without being toxic. The parental molecule, polymyxin B sulphate, is an extremely potent fungicide. The first proposal to produce cell wall mutants with hyper-sensitivity to cytotoxic insult will

be pursued further within the laboratory.

Another refinement would be the functional expression of a human mu class GST. This would provide a member from each of the three classes of human cytosolic GST. This is to be pursued using Polymerase chain reaction (PCR) to produce a full-length mu class cDNA from mRNA purified from a human liver expressing mu class GST.

The system described here also has the potential to be used as a rapid screen for novel antineoplastic agents which are bioactivated by GST mediated reactions. Or conversely to determine agents which are deactivated by GST such that these are not prescribed in cases where acquired or intrinsic resistance is a problem. An example of the former is seen in the bioactivation of azathiopurine and bleomycin by conjugation to GSH (Arrick & Nathan, 1984). The work presented here using DBE shows that the yeast GST expression system can be used to identify compounds whose toxicity is increased by GST metabolism.

Furthermore, this yeast expression system has the potential to identify anticancer drug substrates for the GST enzymes. This type of information is sadly lacking in the literature. Only a few studies have provided conclusive evidence for the role of GST in the formation of GSH-anticancer drug conjugates (Dulik et al, 1986;

Smith et al, 1989). The fact that S.cerevisiae can be grown in large volumes means that large quantities of the expressed proteins can be produced. The protein can be purified to homogeneity and the formation of GSH conjugates examined in the presence or absence of GST. Alternatively, GSH-conjugate formation could be directly assayed in vivo within the yeast cell. In both cases GSH conjugate formation can be assayed either by mass-spectroscopy or by some other means.

The large quantities of protein that can be produced from this expression system is being used to purify the large amounts of the GST (B₁B₁) protein for X-ray crystallographic studies.

From the above it is obvious that the expression of GST within S.cerevisiae is an attractive system that can hopefully answer many fundamental questions regarding GST. Studies can involve structure (via X-ray crystallography) determination of GST substrates, as well as a rapid screening procedure for novel anticancer drugs or drugs for the treatment of drug-resistant diseases. A very flexible system indeed.

SUMMARY

The work presented in this thesis has shown that the expression of mammalian drug metabolising enzymes in S.cerevisiae represents a powerful model system to dissect the complex biochemical pathways arising from xenobiotic metabolism.

The rat P450IIB1 cDNA has been expressed in a functional form as a representative of the cytochrome P-450 monooxygenase super-gene family. The resultant recombinant strain was then tested to determine its suitability as a short term test organism for the screening of potential mutagens. To test all specificity of the system compounds were chosen where PB-inducible P-450 metabolism had been implicated in the activation mechanism (cyclophosphamide and sterigmatocystin). Other promutagens where PB-inducible P-450 activation was known not to play a role were also chosen (benzo(a)pyrene and β -naphthylamine). Finally 2-acetylaminofluorene and 2-anthramine were tested since the activation mechanism of these compounds was unclear. The results clearly demonstrate that S.cerevisiae strains expressing various cytochrome P-450 isoenzymes represent an attractive alternative to other test systems where exogenous activation systems are required.

Many studies have implicated the glutathione S-transferases in drug resistance although this is mainly through GST over-expression in resistant cell lines.

However, complex changes have been shown to occur in the selection for these resistant lines. These changes include alterations in P-170, DNA repair, DNA topoisomerase II, cytochrome P-450 and UDP-glucouronyl- and sulpho-transferases. Thus it is difficult to delineate the role played by GST over-expression in the resistant phenotype. The work presented here was an attempt to simplify the system under examination. Two human GST CDNAs, encoding GST B₁B₁ and GST pi, were expressed in S.cerevisiae and the cellular response to various cytotoxic agents examined. The expression of the alpha and pi class GSTs in S.cerevisiae was shown to confer resistance to the anticancer drugs adriamycin and chlorambucil. The co-expression of GST B₁B₁ and GST pi was also shown to increase resistance to adriamycin over the strains expressing either GST pi or GST B₁B₁.

In addition the alpha class GST also conferred resistance to CDNB and CHP while the resistance phenotype was shown to be compound specific since no increase in resistance was observed after BCNU exposure (BCNU is detoxified by mu class enzymes). Thus the work presented has unequivocally shown that GST can alter cellular sensitivity to cytotoxic agents and implies that these enzymes will play a direct role in tumour response to antineoplastic therapy.

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APPENDIX

Publications arising from this thesis

Black, S.M., Meehan, R.R., Beggs, J.D., Miles, J.S., Adesnik, M., and Wolf, C.R. (1988). The expression of cytochrome P-450 cDNAs in yeast. *Brit. J. Cancer*. 58 : p236.

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